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(57) Abstract

The present invention provides novel synthetic antigenic peptide epitopes, related to IGF-II-R. These synthetic antigenic peptide epitopes are designed for enhanced binding to MHC molecules, and have enhanced immunoregulatory properties relative to their natural counterparts. The synthetic antigenic peptide epitopes of the invention are useful in a variety of methods of modulating an immune response to the synthetic antigenic peptide epitopes and thus to the corresponding native antigenic determinant. Synthetic antigenic peptide epitopes of the invention thus find application in a wide variety of immunomodulatory protocols, including methods to induce or increase an immune response, as well as in methods to suppress or reduce an undesirable immune response, to a corresponding natural epitope.

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PEPTIDES RELATED TO AN IGF-II-R EPITOPE, POLYNUCLEOTIDES ENCODING THE PEPTIDES, AND THEIR USE IN IMMUNOMODULATION

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U. S. Provisional Application Nos. 60/103,229; 60/114,811; 60/120,001; 60/120,002, filed October 5, 1998; January 5, 1999; February 11, 1999; and February 11, 1999, respectively, the contents of which are incorporated herein by reference.

TECHNICAL FIELD

This invention relates to the field of antigenic epitopes, and more particularly to synthetic antigenic peptide epitopes and methods of modulating antigen-specific immune responses.

BACKGROUND OF THE INVENTION

The mammalian immune system is capable of generating responses to foreign antigens, to self antigens present on cancerous cells, and to self antigens present on normal tissues. The immune system comprises two types of antigen-specific cells: B cells and T cells. B cells synthesize both membrane-bound and secreted antibody. T cells can be characterized phenotypically by the manner in which they recognize antigen, by their cell surface markers, and by their secreted products. T cells express distinctive membrane molecules. Included among these are the T cell antigen receptor (TCR), which appears on the cell surface in association with CD3; and accessory molecules such as CD5, CD28 and CD45R. Subpopulations of T cells can be distinguished by the presence of additional membrane molecules. Thus, for example, T cells that express CD4 recognize antigen associated with class II MHC molecules and generally function as helper cells whose roles include enhancement of antibody production by B cells, while T cells that express CD8 recognize antigen associated with class I MHC molecules and generally function as cytotoxic cells.

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Immune cells recognize discrete sites, known as epitopes or antigenic determinants, on the antigen. Epitopes are regions of an immunogen or antigen that bind to antigen-specific membrane-bound receptors on immune cells or to their soluble counterparts, such as antibodies. Both membrane-bound antibody on the surface of a B lymphocyte and secreted antibody recognize soluble antigen. Unlike B cells, which recognize soluble antigen, T cells recognize antigen only when the antigen is associated with self major histocompatibility complex (MHC) gene products on the surface of an antigen presenting cell. This antigen can be displayed together with MHC molecules on the surface of antigen-presenting cells or on virus-infected cells, cancer cells, and grafts.

Disease states can result from invasion by a pathogenic organisms, including bacterial, viral, and protozoan pathogens, and subsequent inefficient or ineffective immune response to the invader. Disease states can also result from the activation of self-reactive T lymphocytes, from the activation of T lymphocytes that provoke allergic reactions, or from the activation of autoreactive T lymphocytes following certain bacterial and parasitic infections, which can produce antigens that mimic human protein, rendering these protein "autoantigens." These diseases include, but are not limited to, the autoimmune diseases, autoimmune disorders that occur as a secondary event to infection with certain bacteria or parasites, T cell mediated allergies, and certain skin diseases such as psoriasis and vasculitis. Furthermore, undesired rejection of a foreign antigen can result in graft rejection or even infertility, and such rejection may be due to activation of specific T lymphocyte populations.

Foreign antigens include macromolecules associated with pathogens such as bacteria, viruses, and protozoans; allergens; and allografts.

Self antigens, under normal physiological conditions, are usually non-immunogenic. However, self antigens can also be immunogenic, as is the case with autoimmune diseases. Autoimmune diseases affect approximately 5% of adults in Europe and North America, often causing chronic debilitating illnesses. Steinman (1993) Scientific American **269**:107-114. Autoimmunity is

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characterized by activation of auto-reactive clones of T or B cells, generating humoral or cell-mediated responses against self-antigens.

Tumor cell antigens are also self antigens, and frequently do not elicit an immune response that results in elimination of the cancerous cells effective to control or eliminate the disease.

In addition, there are other, specific situations in which induction of an immune response to a self antigen is desirable. These include the induction of an immune response to certain antigens as a means of contraception.

The introduction into an animal of an antigen has been widely used for the purposes of modulating the immune response, or lack thereof, to the antigen for a variety of purposes. These include vaccination against pathogens, induction of an immune response to a cancerous cell, reduction of an allergic response, reduction of an immune response to a self antigen that occurs as a result of an autoimmune disorder, reduction of allograft rejection, and induction of an immune response to a self antigen for the purpose of contraception.

Enhancement of the speed and affinity of an immune response to a foreign antigen is the basis of vaccination protocols in which a killed, or live attenuated pathogen, or an antigenic part of a pathogen, is introduced into an individual. One significant disadvantage of such vaccines is that they pose an inherent threat that the virus is not sufficiently attenuated or killed. There is thus the potential for the vaccine to cause the disease against which protection is sought.

In the treatment of cancer, a variety of immunotherapeutic approaches have been taken to generate populations of cytotoxic T lymphocytes which specifically recognize and lyse tumor cells. Many of these approaches depend in part on identifying and characterizing tumor-specific antigens.

More recently, certain pathogen- and tumor-related proteins have been immunologically mimicked with synthetic peptides whose amino acid sequence corresponds to that of an antigenic determinant domain of the pathogen- or tumor-related protein. Despite these advances, peptide immunogens based on native sequences generally perform less than optimally with respect to inducing an immune response. Thus, a need exists for modified synthetic antigenic peptide

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epitopes with enhanced immunomodulatory properties. This invention satisfies this need and provides related advantages as well.

All publications, patents and patent applications cited herein are hereby incorporated by reference into this disclosure in their entirety.

DISCLOSURE OF THE INVENTION

The present invention provides novel synthetic antigenic peptide epitopes. These synthetic antigenic peptide epitopes are designed for enhanced binding to MHC molecules, and have enhanced immunoregulatory properties relative to their natural counterparts. The synthetic antigenic peptide epitopes of the invention are useful in a variety of methods of modulating an immune response to the synthetic antigenic peptide epitopes and thus to the corresponding native antigenic determinant. Synthetic antigenic peptide epitopes of the invention thus find application in a wide variety of immunomodulatory protocols, including methods to induce or increase an immune response, as well as in methods to suppress or reduce an undesirable immune response, to a corresponding natural epitope.

In some embodiments, the synthetic antigenic peptide epitopes of the invention correspond to tumor antigens, and are thus useful as vaccines against tumor cells expressing cell surface tumor antigen. In one of these embodiments, the synthetic antigenic peptide epitopes correspond to an insulin-like growth factor receptor (IGF-II-R). In another embodiment, the present invention provides novel synthetic antigenic gp 100 peptide epitopes which correspond to epitopes found on human melanoma cells. In other embodiments, the synthetic antigenic peptide epitopes of the invention correspond to self antigens on normal (i.e., non-cancerous) tissues. In other embodiments, the synthetic and modified synthetic antigenic peptide epitopes of the invention correspond to foreign (non-self) antigens, such as those associated with organisms such as pathogenic bacteria, viruses and protozoans. In still other embodiments, the peptides correspond to antigens present on allografts which mediate their rejection.

The invention further provides specific structural motifs, or agretopes, which are useful in the rational design of novel synthetic peptides of the invention.

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Polypeptides comprising peptides of the invention, methods for making the peptides and polypeptides, polynucleotides encoding the peptides and polypeptides, and host cells comprising the peptides, polypeptides, and polynucleotides of the invention, are further provided. Further provided are gene delivery vehicles comprising a polynucleotide of the invention. Also provided are compositions comprising the peptides, polypeptides, polynucleotides, and host cells of the invention.

This invention also provides antigen-presenting matrices, which may be antigen presenting cells (APCs), that present the novel synthetic peptides of the invention on their surface, compositions comprising the antigen-presenting matrices, and use of the matrices in immunomodulatory methods, such as cancer therapy. Immune effector cells expanded in the presence of the antigen-presenting matrices are further provided herein, as are compositions comprising these effector cells. These compositions are useful as tumor vaccines and in adoptive immunotherapy.

In another aspect, the invention provides methods of modulating an immune response in an individual to a synthetic antigenic peptide epitope of the invention, and thus to the corresponding natural epitope. In some embodiments, methods are provided for reduction or suppression of an undesired immune response. These methods generally involve introduction into an individual an effective amount of a peptide, or a polynucleotide encoding the peptide, of the invention, in a formulation that results in reduced immunoreactivity to the peptide, and to the native epitope.

In other embodiments, methods are provided for inducing an immune response to a synthetic peptide of the invention. In some of these embodiments, the methods comprise administering to the individual an effective amount of a synthetic peptide of the invention, under conditions that induce an immune response to the peptide. For example, an immune response to a synthetic peptide of the invention can delay or inhibit the formation of a tumor expressing on its surface an epitope corresponding to a synthetic peptide epitope of the invention. In other embodiments, the methods comprise administering to the individual an

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effective amount of a polynucleotide of the invention, such that the polynucleotide enters a cell and a polypeptide of the invention is thereby expressed. In another of these embodiments, the invention provides methods of inducing an immune response to a tumor antigen in an individual, comprising administering to the individual an effective amount of an APC of the invention, under conditions that induce an immune response to the antigen. In a further embodiment, the invention provides methods of adoptive immunotherapy, comprising administering to an individual an effective amount of a population of educated, antigen-specific immune effector cells, specific for a polypeptide of the invention.

In a further aspect, diagnostic methods using the synthetic antigenic peptides of the invention are provided. Methods are provided for detecting the presence of an immune effector cell and/or an immune effector molecule which specifically binds a peptide of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an autoradiograph depicting the results of Northern blot analysis of RNA isolated from tumor cell lines A375, Mel 624, and Mel 1300, and probed with a gp100 cDNA probe.

Figure 2 is a graph of a CTL Assay depicting lysis of A375 (circles), Mel 624 (diamonds), and Mel 1300 (squares) cells by TIL1520 cells at various effector to target cell ratios.

MODES FOR CARRYING OUT THE INVENTION

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual," second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell

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Culture" (R.I. Freshney, ed., 1987); the series "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction," (Mullis et al., eds., 1994); "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

Definitions

As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

As used herein a second polynucleotide "corresponds to" another (a first) polynucleotide if it is related to the first polynucleotide by any of the following relationships:

1) The second polynucleotide comprises the first polynucleotide and the second polynucleotide encodes a gene product.
2) The second polynucleotide is 5' or 3' to the first polynucleotide in cDNA, RNA, genomic DNA, or fragment of any of these polynucleotides. For example, a second polynucleotide may be a

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fragment of a gene that includes the first and second polynucleotides. The first and second polynucleotides are related in that they are components of the gene coding for a gene product, such as a protein or antibody. However, it is not necessary that the second polynucleotide comprises or overlaps with the first polynucleotide to be encompassed within the definition of "corresponding to" as used herein. For example, the first polynucleotide may be a fragment of a 3' untranslated region of the second polynucleotide, for example a promoter sequence. The first and second polynucleotide may be fragment of a gene coding for a gene product. The second polynucleotide may be an exon of the gene while the first polynucleotide may be an intron of the gene.

3) The second polynucleotide is the complement of the first polynucleotide.

The "genotype" of a cell refers to the genetic makeup of the cell and/or its gene expression profile. Modulation of the genotype of a cell can be achieved by introducing additional DNA or RNA either as episomes or as an integral part of the chromosomal DNA of the recipient cell. The genotype can also be modulated by altering the expression level, e.g. mRNA abundance, of a particular gene using agents that regulate gene expression.

A "database" denotes a set of stored data which represent a collection of sequences including nucleotide and peptide sequences, which in turn represent a collection of biological reference materials.

A "native" or "natural" antigen is a polypeptide, protein or a fragment which contains an epitope, which has been isolated from a natural biological source, and which can specifically bind to an antigen receptor, in particular a T cell antigen receptor (TCR), in a subject.

The term "peptide" is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunit may be linked by other bonds, *e.g.* ester, ether, etc. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids,

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including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein. Throughout this specification, numbering of amino acids in a peptide or polypeptide is from amino terminus to carboxy terminus.

The term "sequence motif" refers to a pattern present in a group of molecules. For instance, in one embodiment, the present invention provides for identification of a sequence motif among peptides. In this embodiment, a typical pattern may be identified by characteristic amino acid residues, such as hydrophobic, hydrophilic, basic, acidic, and the like.

A "native" or "natural" antigen is a polypeptide, protein or a fragment which contains an epitope, which has been isolated from a natural biological source, and which can specifically bind to an antigen receptor, in particular a T cell antigen receptor (TCR), in a subject.

A synthetic peptide of the invention is said to "correspond" to a native epitope if the peptide binds to the same TCR as the natural epitope. In some embodiments, a peptide of the invention increases or decreases an immune response specific to the native epitope.

The term "antigen" is well understood in the art and includes substances which are immunogenic, i.e., immunogens, as well as substances which induce immunological unresponsiveness, or anergy, i.e., anergens.

The terms "polynucleotide" and "nucleic acid molecule" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides, and/or their analogs. Nucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes, for example, single-, double-stranded and triple helical molecules, a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of

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any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A nucleic acid molecule may also comprise modified nucleic acid molecules.

"Oligonucleotide" refers to polynucleotides of between about 5 and about 100 nucleotides of single- or double-stranded DNA. Oligonucleotides are also known as oligomers or oligos and may be isolated from genes, or chemically synthesized by methods known in the art.

The term "genetically modified" means containing and/or expressing a foreign gene or nucleic acid sequence which in turn, modifies the genotype or phenotype of the cell or its progeny. In other words, it refers to any addition, deletion or disruption to a cell's endogenous nucleotides.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eukaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook, et al. (1989) *Supra*). Similarly, an eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described below for constructing vectors in general.

"Under transcriptional control" is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. "Operably linked" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

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A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

A "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors and the like. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, retroviral vector refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form which integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a transgene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of

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viruses, including over 50 serotypes. (see, *e.g.*, WO 95/27071). Ads are easy to grow and do not require integration into the host cell genome. Recombinant Ad-derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. (see, WO 95/00655; WO 95/11984). Wild-type AAV has high infectivity and specificity integrating into the host cell's genome. Hermonat and Muzyczka (1984) Proc. Natl. Acad. Sci. USA **81**:6466-6470; Lebkowski et al. (1988) Mol. Cell. Biol. **8**:3988-3996.

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

Gene delivery vehicles also include several non-viral vectors, including DNA/liposome complexes, and targeted viral protein-DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, e.g., TCR, CD3 or CD4.

"Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any

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combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

Examples of stringent hybridization conditions include: incubation temperatures of about 25°C to about 37°C; hybridization buffer concentrations of about 6 X SSC to about 10 X SSC; formamide concentrations of about 0% to about 25%; and wash solutions of about 6 X SSC. Examples of moderate hybridization conditions include: incubation temperatures of about 40°C to about 50°C; buffer concentrations of about 9 X SSC to about 2 X SSC; formamide concentrations of about 30% to about 50%; and wash solutions of about 5 X SSC to about 2 X SSC. Examples of high stringency conditions include: incubation temperatures of about 55°C to about 68°C; buffer concentrations of about 1 X SSC to about 0.1 X SSC; formamide concentrations of about 55% to about 75%; and wash solutions of about 1 X SSC, 0.1 X SSC, or deionized water. In general, hybridization incubation times are from 5 minutes to 24 hours, with 1, 2, or more washing steps, and wash incubation times are about 1, 2, or 15 minutes. SSC is 0.15 M NaCl and 15 mM citrate buffer. It is understood that equivalents of SSC using other buffer systems can be employed.

A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. Preferably, default parameters are used for alignment. A preferred alignment program is BLAST, using default parameters. In particular, preferred programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB +

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GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST.

The terms "major histocompatibility complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC complex is also known as the HLA complex. The proteins encoded by the MHC complex are known as "MHC molecules" and are classified into class I and class II MHC molecules. Class I MHC molecules include membrane heterodimeric proteins made up of an α chain encoded in the MHC associated noncovalently with β2-microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to CD8+ T cells. Class I molecules include HLA-A, -B, and -C in humans. Class I molecules generally bind peptides 8-10 amino acids in length. Class II MHC molecules also include membrane heterodimeric proteins consisting of noncovalently associated α and β chains. Class II MHC are known to participate in antigen presentation to CD4+ T cells and, in humans, include HLA-DP, -DQ, and DR. Class II molecules generally bind peptides 12-20 amino acid residues in length. The term "MHC restriction" refers to a characteristic of T cells that permits them to recognize antigen only after it is processed and the resulting antigenic peptides are displayed in association with either a self class I or class II MHC molecule. Methods of identifying and comparing MHC are well known in the art and are described in Allen et al. (1994) Human Imm. **40**:25-32; Santamaria et al. (1993) Human Imm. **37**:39-50 and Hurley et al. (1997) Tissue Antigens **50**:401-415.

The term "antigen-presenting matrix," as used herein, intends a molecule or molecules which can present antigen in such a way that the antigen can be bound by a T-cell antigen receptor on the surface of a T cell. An antigen-presenting matrix can be on the surface of an antigen-presenting cell (APC), on a vesicle preparation of an APC, or can be in the form of a synthetic matrix on a solid support such as a bead or a plate. An example of a synthetic antigen-presenting matrix is purified MHC class I molecules complexed to β2-

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microglobulin, or purified MHC Class II molecules, or functional portions thereof, attached to a solid support.

The term "antigen presenting cell," as used herein, intends any cell which presents on its surface an antigen in association with a major histocompatibility complex molecule, or portion thereof, or, alternatively, one or more non-classical MHC molecules, or a portion thereof. Examples of suitable APCs are discussed in detail below and include, but are not limited to, whole cells such as macrophages, dendritic cells, B cells, hybrid APCs, and foster antigen presenting cells. Methods of making hybrid APCs have been described. see, for example, International Patent Application No. WO 98/46785; and WO 95/16775.

Dendritic cells (DCs) are potent antigen-presenting cells. It has been shown that DCs provide all the signals required for T cell activation and proliferation. These signals can be categorized into two types. The first type, which gives specificity to the immune response, is mediated through interaction between the T-cell receptor/CD3 ("TCR/CD3") complex and an antigenic peptide presented by a major histocompatibility complex ("MHC") class I or II protein on the surface of APCs. This interaction is necessary, but not sufficient, for T cell activation to occur. In fact, without the second type of signals, the first type of signals can result in T cell anergy. The second type of signals, called co-stimulatory signals, is neither antigen-specific nor MHC-restricted, and can lead to a full proliferation response of T cells and induction of T cell effector functions in the presence of the first type of signals. As used herein, "dendritic cell" is to include, but not be limited to a pulsed dendritic cell, a foster cell or a dendritic cell hybrid.

"Co-stimulatory molecules" are involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. Research accumulated over the past several years has demonstrated convincingly that resting T cells require at least two signals for induction of cytokine gene expression and proliferation (Schwartz (1990) Science **248**:1349-1356; Jenkins (1992) Immunol. Today **13**:69-73). One signal, the one that confers specificity, can be produced by interaction of the TCR/CD3 complex with an

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appropriate MHC/peptide complex. The second signal is not antigen specific and is termed the "co-stimulatory" signal. This signal was originally defined as an activity provided by bone-marrow-derived accessory cells such as macrophages and dendritic cells, the so called "professional" APCs. Several molecules have been shown to enhance co-stimulatory activity. These are heat stable antigen (HSA) (Liu et al. (1992) J. Exp. Med. **175**:437-445), chondroitin sulfate-modified MHC invariant chain (Ii-CS) (Naujokas et al. (1993) Cell 74:257-268), intracellular adhesion molecule 1 (ICAM-1) (Van Seventer (1990) J. Immunol. **144**:4579-4586), B7-1, and B7-2/B70 (Schwartz (1992) Cell **71**:1065-1068). These molecules each appear to assist co-stimulation by interacting with their cognate ligands on the T cells. Co-stimulatory molecules mediate co-stimulatory signal(s) which are necessary, under normal physiological conditions, to achieve full activation of naïve T cells. One exemplary receptor-ligand pair is the B7 co-stimulatory molecule on the surface of APCs and its counter-receptor CD28 or CTLA-4 on T cells (Freeman et al. (1993) Science **262**:909-911; Young et al. (1992) J. Clin. Invest. **90**: 229; Nabavi et al. (1992) Nature **360**:266-268). Other important co-stimulatory molecules are CD40, CD54, CD80, CD86. The term "co-stimulatory molecule" encompasses any single molecule or combination of molecules which, when acting together with a peptide/MHC complex bound by a TCR on the surface of a T cell, provides a co-stimulatory effect which achieves activation of the T cell that binds the peptide. The term thus encompasses B7, or other co-stimulatory molecule(s) on an antigen-presenting matrix such as an APC, fragments thereof (alone, complexed with another molecule(s), or as part of a fusion protein) which, together with peptide/MHC complex, binds to a cognate ligand and results in activation of the T cell when the TCR on the surface of the T cell specifically binds the peptide. Co-stimulatory molecules are commercially available from a variety of sources, including, for example, Beckman Coulter. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified co-stimulatory molecules (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

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As used herein, "solid phase support" or "solid support," used interchangeably, is not limited to a specific type of support. Rather a large number of supports are available and are known to one of ordinary skill in the art. Solid phase supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, alumina gels. As used herein, "solid support" also includes synthetic antigen-presenting matrices, cells, and liposomes. A suitable solid phase support may be selected on the basis of desired end use and suitability for various protocols. For example, for peptide synthesis, solid phase support may refer to resins such as polystyrene (e.g., PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Biosearch, California).

The term "immunomodulatory agent," as used herein, is a molecule, a macromolecular complex, or a cell that modulates an immune response and encompasses a synthetic antigenic peptide of the invention alone or in any of a variety of formulations described herein; a polypeptide comprising a synthetic antigenic peptide of the invention; a polynucleotide encoding a peptide or polypeptide of the invention; a synthetic antigenic peptide of the invention bound to a Class I or a Class II MHC molecule on an antigen-presenting matrix, including an APC and a synthetic antigen-presenting matrix (in the presence or absence of co-stimulatory molecule(s)); a synthetic antigenic peptide of the invention covalently or non-covalently complexed to another molecule(s) or macromolecular structure; and an educated, antigen-specific immune effector cell which is specific for a peptide of the invention.

The term "modulate an immune response" includes inducing (increasing, eliciting) an immune response; and reducing (suppressing) an immune response. An immunomodulatory method (or protocol) is one that modulates an immune response in a subject.

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As used herein, the term "inducing an immune response in a subject" is a term well understood in the art and intends that an increase of at least about 2-fold, more preferably at least about 5-fold, more preferably at least about 10-fold, more preferably at least about 100-fold, even more preferably at least about 500-fold, even more preferably at least about 1000-fold or more in an immune response to an antigen (or epitope) can be detected (measured), after introducing the antigen (or epitope) into the subject, relative to the immune response (if any) before introduction of the antigen (or epitope) into the subject. An immune response to an antigen (or epitope), includes, but is not limited to, production of an antigen-specific (or epitope-specific) antibody, and production of an immune cell expressing on its surface a molecule which specifically binds to an antigen (or epitope). Methods of determining whether an immune response to a given antigen (or epitope) has been induced are well known in the art. For example, antigen-specific antibody can be detected using any of a variety of immunoassays known in the art, including, but not limited to, ELISA, wherein, for example, binding of an antibody in a sample to an immobilized antigen (or epitope) is detected with a detectably-labeled second antibody (e.g., enzyme-labeled mouse anti-human Ig antibody). Immune effector cells specific for the antigen can be detected any of a variety of assays known to those skilled in the art, including, but not limited to, FACS, or, in the case of CTLs, ^{51}Cr-release assays, or ^{3}H-thymidine uptake assays.

The term "immune effector cells" refers to cells capable of binding an antigen or which mediate an immune response. These cells include, but are not limited to, T cells, B cells, monocytes, macrophages, NK cells and cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates. Certain diseased tissue expresses specific antigens and CTLs specific for these antigens have been identified. For example, approximately 80% of melanomas express the antigen known as gp100.

The term "immune effector molecule," as used herein, refers to molecules capable of antigen-specific binding, and includes antibodies, T cell antigen receptors, and MHC Class I and Class II molecules.

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A "naïve" immune effector cell is an immune effector cell that has never been exposed to an antigen.

As used herein, the term "educated, antigen-specific immune effector cell," is an immune effector cell as defined above, which has encountered antigen and which is specific for that antigen. An educated, antigen-specific immune effector cell may be activated upon binding antigen. "Activated" implies that the cell is no longer in G_0 phase, and begins to produce cytokines characteristic of the cell type. For example, activated CD4+ T cells secrete IL-2 and have a higher number of high affinity IL-2 receptors on their cell surfaces relative to resting CD4+ T cells.

A peptide or polypeptide of the invention may be preferentially recognized by antigen-specific immune effector cells, such as B cells and T cells. In the context of T cells, the term "recognized" intends that a peptide or polypeptide of the invention, comprising one or more synthetic antigenic epitopes, is recognized, i.e., is presented on the surface of an APC together with (i.e., bound to) an MHC molecule in such a way that a T cell antigen receptor (TCR) on the surface of an antigen-specific T cell binds to the epitope wherein such binding results in activation of the T cell. The term "preferentially recognized" intends that a polypeptide of the invention is substantially not recognized, as defined above, by a T cell specific for an unrelated antigen. Assays for determining whether an epitope is recognized by an antigen-specific T cell are known in the art and are described herein.

The term "autogeneic," or "autologous," as used herein, indicates the origin of a cell. Thus, a cell being administered to an individual (the "recipient") is autogeneic if the cell was derived from that individual (the "donor") or a genetically identical individual. An autogeneic cell can also be a progeny of an autogeneic cell. The term also indicates that cells of different cell types are derived from the same donor or genetically identical donors. Thus, an effector cell and an antigen presenting cell are said to be autogeneic if they were derived from the same donor or from an individual genetically identical to the donor, or if they are progeny of cells derived from the same donor or from an individual genetically identical to the donor.

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Similarly, the term "allogeneic," as used herein, indicates the origin of a cell. Thus, a cell being administered to individual (the "recipient") is allogeneic if the cell was derived from an individual not genetically identical to the recipient; in particular, the term relates to non-identity in expressed MHC molecules. An allogeneic cell can also be a progeny of an allogeneic cell. The term also indicates that cells of different cell types are derived from genetically non-identical donors, or if they are progeny of cells derived from genetically non-identical donors. For example, an APC is said to be allogeneic to an effector cell if they are derived from genetically non-identical donors.

As used herein, the term "a disease or condition related to a population of $CD4^+$ or $CD8^+$ T cells" is one which can be related to a population of $CD4^+$ or $CD8^+$ T cells, such that these cells are primarily responsible for the pathogenesis of the disease; it is also one in which the presence of $CD4^+$ or $CD8^+$ T cells is an indicia of a disease state; it is also one in which the presence of a population $CD4^+$ or $CD8^+$ T cells is not the primary cause of the disease, but which plays a key role in the pathogenesis of the disease; it is also one in which a population of $CD4^+$ or $CD8^+$ T cells mediates an undesired rejection of a foreign antigen. Examples of a condition related to a population of $CD4^+$ or $CD8^+$ T cells include, but are not limited to, autoimmune disorders, graft rejection, immunoregulatory disorders, and anaphylactic disorders.

As used herein, the terms "neoplastic cells," "neoplasia," "tumor," "tumor cells," "cancer" and "cancer cells," (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with educated, antigen-specific immune effector cells described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^3H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the

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following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (morphologically, genetically, or phenotypically) to the parent cell. By "expanded" is meant any proliferation or division of cells.

As used herein, the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or proliferation. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1I), interleukin-11 (IL-11), MIP-1I, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO) and flt3 ligand. The present invention also includes culture conditions in which one or more cytokine is specifically excluded from the medium. Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

A "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative." For example, where the purpose of the experiment is to determine a correlation of an altered expression level of a gene with a particular type of cancer, it is generally preferable to use a positive control (a subject or a sample from a subject, carrying

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such alteration and exhibiting syndromes characteristic of that disease), and a negative control (a subject or a sample from a subject lacking the altered expression and clinical syndrome of that disease).

"PCR primers" refer to primers used in "polymerase chain reaction" or "PCR," a method for amplifying a DNA base sequence using a heat-stable polymerase such as Taq polymerase, and two oligonucleotide primers, one complementary to the (+)-strand at one end of the sequence to be amplified and the other complementary to the (-)-strand at the other end. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation produce exponential and highly specific amplification of the desired sequence. (See, e.g., PCR 2: A PRACTICAL APPROACH, *Supra*). PCR also can be used to detect the existence of the defined sequence in a DNA sample.

"Host cell" or "recipient cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of exogenous nucleic acid molecules, polynucleotides and/or peptides (or polypeptides). It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. The cells may be prokaryotic or eukaryotic, and include but are not limited to bacterial cells, yeast cells, animal cells, and mammalian cells, e.g., murine, rat, simian or human.

An "antibody" is an immunoglobulin molecule capable of binding an antigen. As used herein, the term encompasses not only intact immunoglobulin molecules, but also anti-idiotypic antibodies, mutants, fragments, fusion proteins, bi-specific antibodies, humanized proteins and modifications of the immunoglobulin molecule that comprise an antigen recognition site of the required specificity.

An "antibody complex" is the combination of antibody (as defined above) and its binding partner or ligand.

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The term "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. For example, with respect to a polynucleotide, an isolated polynucleotide is one that is separated from the 5' and 3' sequences with which it is normally associated in the chromosome. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, does not require "isolation" to distinguish it from its naturally occurring counterpart. In addition, a "concentrated," "separated" or "diluted" polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof , which differs from the naturally occurring counterpart in its primary sequence or for example, by its glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally occurring counterpart by its primary sequence, or alternatively, by another characteristic such as glycosylation pattern. Although not explicitly stated for each of the inventions disclosed herein, it is to be understood that all of the above embodiments for each of the compositions disclosed below and under the appropriate conditions, are provided by this invention. Thus, a non-naturally occurring polynucleotide is provided as a separate embodiment from the isolated naturally occurring polynucleotide. A protein produced in a bacterial cell is provided as a separate embodiment from the naturally occurring protein isolated from a eukaryotic cell in which it is produced in nature.

An "enriched" population of cells, as used herein, means that a cell population is at least about 50-fold, more preferably at least about 500-fold, and even more preferably at least about 5000-fold or more enriched from an original naive cell population. The proportion of the enriched cell population which comprises antigen-specific cells can vary substantially, from less than 10% up to 100% antigen-specific cells.

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An "isolated" population of cells is "substantially free" of cells and materials with which it is associated in nature. By "substantially free" or "substantially pure" means at least 50% of the population are the desired cell type, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90%.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent , solid support or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages. In the context of a disease state, an effective amount of an immunomodulatory agent of the invention, including a peptide of the invention, a polynucleotide of the invention, an educated, antigen-specific immune effector cell and/or an APC and/or synthetic antigen-presenting matrix of the invention, is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

Synthetic antigenic peptide epitopes of the invention

This invention provides novel, synthetic antigenic peptide sequences which are useful in a variety of methods for modulating an immune response to a peptide epitope. The synthetic antigenic peptide epitope sequences of the present invention differ from their natural counterparts in at that they contain alterations in

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amino acid sequence, relative to the native sequence, in MHC Class I or II binding domains, thereby conferring tighter binding to the MHC. They may optionally further contain mutations, for example, in the putative T cell receptor-binding domain, resulting in an apparent increased affinity for the T cell antigen receptor. Alternatively, the peptides contain mutations that do not confer tighter binding to the MHC but still result in increased avidity for the T cell receptor. These differences from the native sequence confer advantages in the methods of the present invention over the native sequence, in that the synthetic antigenic peptide epitopes of the invention have enhanced immunomodulatory properties.

Further encompassed by the term "synthetic antigenic peptide" are multimers (concatemers) of a synthetic antigenic peptide of the invention, optionally including intervening amino acid sequences.

In some embodiments, a synthetic antigenic peptide of the invention binds to an MHC Class I or Class II molecule with a higher affinity that the "natural" or "native" sequence. In these embodiments, a synthetic antigenic peptide of the invention binds to an MHC Class I or Class II molecule with an affinity at least about 2-fold higher, preferably at least about 10-fold higher, more preferably at least about 50-fold higher, more preferably at least about 100-fold higher, more preferably at least about 200-fold higher, more preferably at least about 500-fold higher, more preferably at least about 1000-fold higher, even more preferably at least about 2000-fold or more higher than the native sequence. Methods for determining binding affinity to an MHC Class I or Class II molecule are known in the art and include, but are not limited to, calculating the affinity based on an algorithm (see, for example, Parker et al. (1992) J. Immunol. **149**:3580-3587); and experimentally determining binding affinity (see, for example, Tan et al. (1997) J. Immunol. Meth. **209(1)**:25-36. For example, the relative binding of a peptide to a Class I molecule can be measured on the basis of binding of a radiolabeled standard peptide to detergent-solubilized MHC molecules, using various concentrations of test peptides (e.g., ranging from 100 mM to 1nM). MHC Class I heavy chain and β2-microglobulin are coincubated with a fixed concentration (e.g., 5 nM) radiolabeled standard (control) peptide and various concentrations of

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a test peptide for a suitable period of time (e.g., 2 hours to 72 hours) at room temperature in the presence of a mixture of protease inhibitors. A control tube contains standard peptide and MHC molecules, but no test peptide. The percent MHC-bound radioactivity is determined by gel filtration. The IC50 (concentration of test peptide which results in 50% inhibition of binding of control peptide) is calculated for each peptide.

In some embodiments, a synthetic peptide of the invention binds to a TCR with a higher affinity that the "natural" sequence. In these embodiments, a synthetic peptide of the invention binds to a TCR with an affinity at least about 2-fold higher, preferably at least about 10-fold higher, more preferably at least about 50-fold higher, more preferably at least about 100-fold higher, more preferably at least about 200-fold higher, more preferably at least about 500-fold higher, more preferably at least about 1000-fold higher, even more preferably at least about 2000-fold or more higher than the native epitope. Methods for determining binding affinity to a TCR are known in the art and include, but are not limited to, those described in al-Ramadi et al. (1992) J. Immunol. **155(2)**:662-673; and Zuegel et al. (1998) J. Immunol. **161(4)**:1705-1709.

Further provided by the present invention are isolated polypeptides comprising synthetic antigenic peptide amino acid sequences of the invention.

Synthetic antigenic peptide epitopes of the present invention can be designed based on known amino acid sequences of antigenic peptide epitopes.

The protein antigens of a number of pathogens, cancerous cells, and self tissues are known from the scientific literature, as are peptides from such antigens that bind to a particular HLA-type. Thus, in some embodiments, synthetic antigenic peptide epitopes of the present invention are modifications of antigenic determinants that have previously been shown to be recognized in connection with a particular MHC molecule by cytotoxic T lymphocytes or $CD4^+$ T cells *in vitro* and/or *in vivo*.

The present invention provides synthetic gp100 antigenic peptide epitopes with modified amino acid sequences relative to their natural counterparts that

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exhibit enhanced binding to MHC molecules, particularly MHC Class I molecules.

Peptide epitopes associated with pathogenic organisms include peptides from the influenza nucleoprotein composed of residues 365-80 (NP365-80), NP50-63, and NP147-58 and peptides from influenza hemagglutinin HA202-21 and HA523-45, defined previously in class I restricted cytotoxicity assays. Perkins et al. (1989) J. Exp. Med. **170**: 279-289. Enhanced efficiency of association of such polypeptides to specific class I molecules on antigen presenting cells *in vivo* has major implications for the use of these synthetic peptides as influenza vaccines. Other examples of synthetic peptides containing known epitopes that can be recognized by MHC-restricted CTLs include influenza strain A/Jap/57 hemagglutinin protein, residues 508-530; influenza strain A/PR8/34 nucleoprotein residues 360-385; HIV Pol (reverse transcriptase) residues 203-219; Sendai virus nucleoprotein peptide, residues 324-332; and the vesicular stomatitis nucleotide protein, amino acid residues 52-59. Peptides representing epitopes displayed by the malarial parasite *Plasmodium falciparum* have been described. U.S. Patent No. 5,609,872. In addition, antigenic determinants of proteins involved in malignant disorders can also be used to protect against malignant disease. Antigenic epitopes associated with malignant cells are known in the art and include the melanoma-associated antigens gp100, MART-1, and tyrosinase.

An example of a self tissue antigen recognized in autoimmune disorders is the acetylcholine receptor (AChR) which is recognized in myasthenia gravis. The T lymphocyte response in these patients may be directed to additional epitopes on the AChR. Although the majority of T cell recognition sites are on the I subunit, T cells also recognize epitopes in the other subunits. Indeed, T cells from patients have been shown to respond to more than 30 different AChR-derived peptides. Examples of AChR epitopes are the following:

1 HM1: Y N L K W N Y N L K W N Y N L K W N (SEQ ID NO:31)

2 HM2: P D D Y G G P D D Y G G P D D Y G G (SEQ ID NO:32)

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HM3: V K K I H I V K K I H I V K K I H I (SEQ ID NO:33)

HM4: K W N P D D K W N P D D K W N P D D Y (SEQ ID NO:34)

HM5: Y G G V K K Y G G V K K Y G G V K K (SEQ ID NO:35)

HM6: W N P D D Y G G V K W N P D D Y G G V K (SEQ ID NO:36)

Another class of self antigens for which antigenic epitopes have been described is human chorionic gonadotropin (hCG) beta subunit. U.S. Patent No. 5,733,553. These epitopes find utility in contraceptive methods.

This list of peptides is exemplary only and is not intended to limit the Class I or Class II peptides that can be modified for use in the methods of the present invention can be employed. Class I and Class II peptides that can be used with the present invention can also be determined empirically in accordance with techniques known in the art. For example, the peptides that are displayed by a variety of different class I molecules can be defined for a given pathogen-related antigen by infecting somatic cells of given class I HLA types with the pathogen of interest. The peptides that bind to the class I molecules after normal intracellular processing are then eluted from the target cell surface and subjected to sequence analysis in accordance with known techniques. Alternatively, overlapping peptides from a given pathogen-related protein can be synthesized and analyzed for their ability to bind to the various Class I and Class II HLA types. Alternatively, a method such as SPHERE, which is described in more detail below, can be used to identify antigenic epitopes.

Production of antigenic peptides and polypeptides comprising antigenic peptides of the invention

The peptides used in accordance with the method of the present invention can be obtained in any one of a number of conventional ways. Because they will generally be short sequences, they can be prepared by chemical synthesis using standard techniques. Particularly convenient are the solid phase peptide synthesis techniques. Automated peptide synthesizers are commercially available, as are the reagents required for their use. Alternatively, the peptides can be prepared by

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enzymatic digestion or cleavage of naturally occurring proteins. The peptides can also be prepared using recombinant techniques known to those of skill in the art.

In one embodiment, isolated peptides of the present invention can be synthesized using an appropriate solid state synthetic procedure. Steward and Young, eds. (1968) SOLID PHASE PEPTIDE SYNTHESIS, Freemantle, San Francisco, Calif. A preferred method is the Merrifield process. Merrifield (1967) Recent progress in Hormone Res. **23**:451. The antigenic activity of these peptides may conveniently be tested using, for example, the assays described herein.

Once an isolated peptide of the invention is obtained, it may be purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. For immunoaffinity chromatography, an epitope may be isolated by binding it to an affinity column comprising antibodies that were raised against that peptide, or a related peptide of the invention, and were affixed to a stationary support.

Alternatively, affinity tags such as hexa-His (Invitrogen), Maltose binding domain (New England Biolabs), influenza coat sequence (Kolodziej et al. (1991) Methods Enzymol. **194**:508-509), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. Isolated peptides can also be physically characterized using such techniques as proteolysis, nuclear magnetic resonance, and x-ray crystallography.

Also included within the scope of the invention are antigenic peptides that are differentially modified during or after translation, e.g., by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand. Ferguson et al. (1988) Ann. Rev. Biochem. **57**:285-320. This is achieved using various chemical methods or by expressing the polynucleotides in different host cells, e.g., bacterial, mammalian, yeast, or insect cells.

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Peptide formulations

A synthetic antigenic peptide epitope of the invention can be used in a variety of formulations, which may vary depending on the intended use.

A synthetic antigenic peptide epitope of the invention can be covalently or non-covalently linked (complexed) to various other molecules, the nature of which may vary depending on the particular purpose. For example, a peptide of the invention can be covalently or non-covalently complexed to a macromolecular carrier, including, but not limited to, natural and synthetic polymers, proteins, polysaccharides, poly(amino acid), polyvinyl alcohol, polyvinyl pyrrolidone, and lipids. A peptide can be conjugated to a fatty acid, for introduction into a liposome. U.S. Patent No. 5,837,249. A synthetic peptide of the invention can be complexed covalently or non-covalently with a solid support, a variety of which are known in the art. A synthetic antigenic peptide epitope of the invention can be associated with an antigen-presenting matrix with or without co-stimulatory molecules, as described in more detail below.

Examples of protein carriers include, but are not limited to, superantigens, serum albumin, tetanus toxoid, ovalbumin, thyroglobulin, myoglobulin, and immunoglobulin.

Peptide-protein carrier polymers may be formed using conventional crosslinking agents such as carbodiimides. Examples of carbodiimides are 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide (CMC), 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC) and 1-ethyl-3-(4-azonia-44-dimethylpentyl) carbodiimide.

Examples of other suitable crosslinking agents are cyanogen bromide, glutaraldehyde and succinic anhydride. In general, any of a number of homobifunctional agents including a homobifunctional aldehyde, a homobifunctional epoxide, a homobifunctional imidoester, a homobifunctional N-hydroxysuccinimide ester, a homobifunctional maleimide, a homobifunctional alkyl halide, a homobifunctional pyridyl disulfide, a homobifunctional aryl halide, a homobifunctional hydrazide, a homobifunctional diazonium derivative and a homobifunctional photoreactive compound may be used. Also included are

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heterobifunctional compounds, for example, compounds having an amine-reactive and a sulfhydryl-reactive group, compounds with an amine-reactive and a photoreactive group and compounds with a carbonyl-reactive and a sulfhydryl-reactive group.

Specific examples of such homobifunctional crosslinking agents include the bifunctional N-hydroxysuccinimide esters dithiobis(succinimidylpropionate), disuccinimidyl suberate, and disuccinimidyl tartarate; the bifunctional imidoesters dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate; the bifunctional sulfhydryl-reactive crosslinkers 1,4-di-[3'-(2'-pyridyldithio) propion-amido]butane, bismaleimidohexane, and bis-N-maleimido-1, 8-octane; the bifunctional aryl halides 1,5-difluoro-2,4-dinitrobenzene and 4,4'-difluoro-3,3'-dinitrophenylsulfone; bifunctional photoreactive agents such as bis-[b-(4-azidosalicylamido)ethyl]disulfide; the bifunctional aldehydes formaldehyde, malondialdehyde, succinaldehyde, glutaraldehyde, and adipaldehyde; a bifunctional epoxide such as 1,4-butaneodiol diglycidyl ether, the bifunctional hydrazides adipic acid dihydrazide, carbohydrazide, and succinic acid dihydrazide; the bifunctional diazoniums o-tolidine, diazotized and bis-diazotized benzidine; the bifunctional alkylhalides N1N'-ethylene-bis(iodoacetamide), N1N'-hexamethylene-bis(iodoacetamide), N1N'-undecamethylene-bis(iodoacetamide), as well as benzylhalides and halomustards, such as a1a'-diiodo-p-xylene sulfonic acid and tri(2-chloroethyl)amine, respectively.

Examples of other common heterobifunctional cross-linking agents that may be used to effect the conjugation of proteins to peptides include, but are not limited to, SMCC succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester), SIAB (N-succinimidyl(4-iodoacteyl)aminobenzoate), SMPB (succinimidyl-4-(p-maleimidophenyl)butyrate), GMBS (N-(γ-maleimidobutyryloxy)succinimide ester), MPBH (4-(4-N-maleimidopohenyl) butyric acid hydrazide), M2C2H (4-(N-maleimidomethyl) cyclohexane-1-carboxyl-hydrazide), SMPT (succinimidyloxycarbonyl-á-methyl-á-(2-pyridyldithio)toluene), and SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate).

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Crosslinking may be accomplished by coupling a carbonyl group to an amine group or to a hydrazide group by reductive amination.

Peptides of the invention also may be formulated as non-covalent attachment of monomers through ionic, adsorptive, or biospecific interactions. Complexes of peptides with highly positively or negatively charged molecules may be done through salt bridge formation under low ionic strength environments, such as in deionized water. Large complexes can be created using charged polymers such as poly-(L-glutamic acid) or poly-(L-lysine) which contain numerous negative and positive charges, respectively. Adsorption of peptides may be done to surfaces such as microparticle latex beads or to other hydrophobic polymers, forming non-covalently associated peptide-superantigen complexes effectively mimicking crosslinked or chemically polymerized protein. Finally, peptides may be non-covalently linked through the use of biospecific interactions between other molecules. For instance, utilization of the strong affinity of biotin for proteins such as avidin or streptavidin or their derivatives could be used to form peptide complexes. These biotin-binding proteins contain four binding sites that can interact with biotin in solution or be covalently attached to another molecule. Wilchek (1988) Anal Biochem. **171**:1-32. Peptides can be modified to possess biotin groups using common biotinylation reagents such as the N-hydroxysuccinimidyl ester of D-biotin (NHS-biotin) which reacts with available amine groups on the protein. Biotinylated peptides then can be incubated with avidin or streptavidin to create large complexes. The molecular mass of such polymers can be regulated through careful control of the molar ratio of biotinylated peptide to avidin or streptavidin.

Also provided by this application are the peptides and polypeptides described herein conjugated to a detectable agent for use in the diagnostic methods. For example, detectably labeled peptides and polypeptides can be bound to a column and used for the detection and purification of antibodies. They also are useful as immunogens for the production of antibodies, as described below.

The peptides of this invention also can be combined with various liquid phase carriers, such as sterile or aqueous solutions, pharmaceutically acceptable

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carriers, suspensions and emulsions. Examples of non-aqueous solvents include propyl ethylene glycol, polyethylene glycol and vegetable oils. When used to prepare antibodies, the carriers also can include an adjuvant that is useful to non-specifically augment a specific immune response. A skilled artisan can easily determine whether an adjuvant is required and select one. However, for the purpose of illustration only, suitable adjuvants include, but are not limited to, Freund's Complete and Incomplete, mineral salts and polynucleotides.

Methods for designing synthetic antigenic peptide epitopes

The synthetic antigenic peptide epitopes can be designed based on natural peptide epitopes identified using any method known in the art. The following provides non-limiting examples of methods which can be used. In addition, modifications or combinations of any of the following methods can be used. For example, modifications of the SAGE and the SPHERE methods are described in International Patent Application No. PCT/US99/01462.

Methods involving isolating and assaying MHC molecules from antigen presenting cells can be used to identify peptides bound to the MHC molecules. Chicz and Urban (1994) Immunol. Today **1-5**:155-160. Bacteriophage "phage display" libraries can also be constructed. Using the "phage method" (Scott and Smith (1990) Science **249**:386-390; Cwirla et al. (1990) Proc. Natl. Acad. Sci. USA **87**:6378-6382; Devlin et al. (1990) Science **249**:404-406), very large libraries can be constructed (10^6-10^8 chemical entities). Other methods to identify peptide epitopes which can be used involve primarily chemical methods, of which the Geysen method (Geysen et al. (1986) Molecular Immunology **23**:709-715; Geysen et al. (1987) J. Immunologic Method **102**:259-274; and the method of Fodor et al. (1991) Science **251**:767-773) are examples. Furka et al. (1988) 14th International Congress of Biochemistry, Volume 5. Abstract FR:013; Furka, (1991) Int. J. Peptide Protein Res. **37**:487-493). Houghton (U.S. Patent No. 4,631,211 issued December 1986) and Rutter et al. (U. S. Patent No. 5,101,175, issued April 23, 1991) describe methods to produce a mixture of peptides that can be tested as agonists or antagonists. Other methods which can be employed

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involve use of synthetic libraries (Needels et al. (1993) Proc. Natl. Acad. Sci. USA **90**:10700-4; Ohlmeyer et al. (1993) Proc. Natl. Acad. Sci. USA **90**:10922-10926; Lam et al., International Patent Publication No. WO 92/00252, each of which is incorporated herein by reference in its entirety), and the like can be used to screen for receptor ligands. Techniques based on cDNA subtraction or differential display have been described amply in the literature and can also be used. see, for example, Hedrick et al. (1984) Nature **308**:149; and Lian and Pardee (1992) Science **257**:967. The expressed sequence tag (EST) approach is a valuable tool for gene discovery (Adams et al. (1991) Science **252**:1651), as are Northern blotting, RNase protection, and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Alwine et al. (1977) Proc. Natl. Acad. Sci. USA **74**:5350; Zinn et al. (1983) Cell **34**:865; Veres et al. (1987) Science **237**:415). Another technique which can be used is the "pepscan" technique (Van der Zee (1989) Eur. J. Immunol. **19**:43-47) in which several dozens of peptides are simultaneously synthesized on polyethylene rods arrayed in a 96-well microliter plate pattern, similar to an indexed library in that the position of each pin defines the synthesis history on it. Peptides are then chemically cleaved from the solid support and supplied to irradiated syngeneic thymocytes for antigen presentation. A cloned CTL line is then tested for reactivity in a proliferation assay monitored by ^{3}H-thymidine incorporation.

Another method which can be used is the SAGE technique, which allows a rapid, detailed analysis of thousands of transcripts. The SAGE method is described in U.S. Patent No. 5,695,937. SAGE is based on two principles. First, a short nucleotide sequence tag (9 to 10 bp) contains sufficient information content to uniquely identify a transcript provided it is isolated from a defined position within the transcript. For example, a sequence as short as 9 bp can distinguish 262,144 transcripts (Fields et al. (1994) Nature Genet. 7:345) given a random nucleotide distribution at the tag site, whereas current estimates suggest that even the human genome only encodes about 80,000 transcripts. Fields et al. (1994) Nature Genet. 7:345. Second, concatenation of short sequence tags allows the efficient analysis of transcripts in a serial manner by sequencing of multiple

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tags within a single clone. As with serial communication by computers, wherein information is transmitted as a continuous string of data, serial analysis of the sequence tags requires a means to establish the register and boundaries of each tag.

The SPHERE method has been previously described. WO 97/35035. The SPHERE approach utilizes combinatorial peptide libraries synthesized on polystyrene beads wherein each bead contains a pure population of a unique peptide that can be chemically released from the beads in discrete aliquots. Released peptide from pooled bead arrays are screened using methods to detect T cell activation, including, for example, ^{3}H-thymidine incorporation (for $CD4^{+}$ or $CD8^{+}$T cells), ^{51}Cr-release assay (for CTLs) or IL-2 production (for $CD4^{+}$ T cells) to identify peptide pools capable of activating a T cell of interest. By utilizing an iterative peptide pool/releasing strategy, it is possible to screen more than 10^{7} peptides in just a few days. Analysis of residual peptide on the corresponding positive beads (>100 pmoles) allows rapid and unambiguous identification of the epitope sequence.

A brief overview of an assay to identify peptides binding to CTLs is as follows: roughly speaking, ten 96-well plates with 1000 beads per well will accommodate 10^{6} beads; ten 96-well plates with 100 beads per well will accommodate 10^{5} beads. In order to minimize both the number of CTL cells required per screen and the amount of manual manipulations, the eluted peptides can be further pooled to yield wells with any desired complexity. For example, based on experiments with soluble libraries, it is possible to screen 10^{7} peptides in 96-well plates (10,000 peptides per well) with as few as 2×10^{6} CTL cells. After cleaving a percentage of the peptides from the beads, incubating them with gamma-irradiated foster APCs and the cloned CTL line(s), positive wells determined by ^{3}H-thymidine incorporation are further examined. Alternatively, as pointed out above, cytokine production or cytolytic ^{51}Cr-release assays may be used. Coulie et al. (1992) Int. J. Cancer **50**:289-291. Beads from each positive well will be separated and assayed individually as before, utilizing an additional percentage of the peptide from each bead. Positive individual beads will then be

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decoded, identifying the reactive amino acid sequence. Analysis of all positives will give a partial profile of conservatively substituted epitopes which stimulate the CTL clone tested. At this point, the peptide can be resynthesized and retested. Also, a second library (of minimal complexity) can be synthesized with representations of all conservative substitutions in order to enumerate the complete spectrum of derivatives tolerated by a particular CTL. By screening multiple CTLs (of the same MHC restriction) simultaneously, the search for crossreacting epitopes is greatly facilitated.

The described method for the identification of $CD8^+$ MHC Class I-restricted CTL epitopes can be applied to the identification of $CD4^+$ MHC Class II-restricted $CD4^+$ T cell epitopes. In this case, MHC Class II allele-specific libraries are synthesized such that haplotype-specific anchor residues are represented at the appropriate positions. MHC Class II agretopic motifs have been identified for the common alleles. Rammensee (1995) Curr. Opin. Immunol. 7:85-96; Altuvia et al. (1994) Mol. Immunol. **24**:375-379; Reay et al. (1994) J. Immunol. **152**:3946-3957; Verreck et al. (1994) Eur. J. Immunol. **24**:375-379; Sinigaglia and Hammer (1994) Curr. Opin. Immunol. **6**:52-56; Rotzschke and Falk (1994) Curr. Opin. Immunol. **6**:45-51. The overall length of the peptides will be 12-20 amino acid residues, and previously described methods may be employed to limit library complexity. The screening process is identical to that described for MHC Class I-associated epitopes except that the antigen presenting matrix would comprise MHC Class II molecules and any required co-stimulatory molecules. MHC Class II molecule-bearing antigen-presenting cells include, but are not limited to, B lymphoblastoid cell lines (B-LCL). As one example, previously characterized B-LCLs that are defective in antigen processing, thus allowing specific presentation of exogenously added antigen, can be employed. Mellins et al. (1991) J. Exp. Med. **174**:1607-1615. The libraries are screened for reactivity with isolated $CD4^+$ MHC Class II allele-specific CD4+ cells. Reactivity may be measured by ^{3}H-thymidine incorporation according to the method of Mellins et al., *Supra*, or by any of the methods previously described for MHC Class I-associated epitope screening.

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Synthetic antigenic peptides

In one embodiment, the invention provides synthetic IGF-II-R antigenic peptides. These peptides differ from the amino acid sequence of the native epitope in that they bind with greater affinity to MHC Class I molecules than a peptide having the amino acid sequence of the native epitope. These peptides are useful in methods of the invention to induce (or enhance, or elicit) an immune response to the peptide. The peptides can generate an immune response not only to themselves, but to the native epitope. CTLs reactive with a peptide of the invention and with the native epitope can suppress the growth of a tumor cell expressing that epitope.

Insulin-like growth factor-II receptor (IGF-IIR) plays an important role in regulating cell growth. It binds to and facilitates the activation of the inactive complex of the growth inhibitor, transforming growth factor α (TGFα), and inactivates the growth stimulator, insulin-like growth factor-II (IGF-II). Dennis et al. (1991) Proc. Natl. Acad. Sci. USA **88**:580-584; De Bleser et al. (1995) Hepatol. **21**:1429-1437; and Kornfeld (1992) Ann. Rev. Biochem. **61**:307-330. IGF-IIR is a single chain transmembrane protein, and includes an extracellular domain which contains 15 homologous repeats, each consisting of 134 to 167 amino acid residues. The nucleotide and translated amino acid sequence of a mouse IGF-IIR cDNA is provided in GenBank Accession No. 1709091. IGF-II plays key roles in mammalian development, influencing fetal cell division and differentiation. The mature 67-amino acid peptide has significant sequence identity with both insulin and IGF-I. O'Dell and Day (1998) Int. J. Biochem. Cell Biol. **30**(7):767-771. IGF-II is expressed in most embryonic tissues, but, in adult tissues, IGF-II expression is generally reduced or undetectable.

The IGF-II gene is often reactivated during tumorigenesis. Duguay et al. (1998) J. Biol. Chem. **273**(29):18443-18451. Indeed, an increase in IGF-II has been associated with tumors such as rhabdomyosarcoma (El-Badry et al. (1990) Cell Growth Diff. **1**:325-3331); gastric cancer (Shiraishi et al. (1998) Int. J. Oncol. **13**(3):519-523); hepatocellular carcinoma (Sohda et al. (1998) J. Hum. Genet.

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43(1):49-53); breast and prostate cancer (Li et al. (1998) Cell Tissue Res. 291(3): 469-472); adrenocortical tumors; and colorectal cancer (Kawamoto et al. (1998) Oncology 55(3):242-248).

An increase in expression of IGF-II-R has been observed in a variety of cancer cells including pancreatic cancer cells (Ishiwata et al. (1997) Pancreas 15(4):367-373); astrocytomas and meningiomas (Antoniades et al. (1992) Int. J. Cancer 50:215-222); and thyroid neoplasms (Yashiro et al. (1991) Eur. J. Cancer 27(6):699-703). Others have observed an increase in cancer cell motility induced by IGF-II, and suggested that increased IGF-II and/or IGF-II-R expression contributes to cancer cell metastasis.

Thus, in some embodiments of the invention, a synthetic antigenic peptide epitope encompasses peptides having, or consisting essentially of, one or more of the following sequences (also shown in Example 1, Table 1): FLFSWYAXV (SEQ ID NO:1); FLFSWFALV (SEQ ID NO:3); FLYSWWAIV (SEQ ID NO:5); FLYSWWWPV (SEQ ID NO:7); and/or FLFLWFFEV (SEQ ID NO:9), wherein X is any amino acid. In some embodiments, X is not a cysteine. The foregoing sequences are referred to herein as synthetic IGF-II-R antigenic peptide epitopes. Also encompassed by "synthetic IGF-II-R antigenic peptide" are peptides having the consensus sequence $FLX_1X_2WX_3X_4X_5V$ (SEQ ID NO:13). Accordingly, the invention further provides synthetic antigenic peptides having the sequence $FLX_1X_2WX_3X_4X_5V$ (SEQ ID NO:13), wherein X_1 is F or Y or other amino acid, X_2 is S or L or other amino acid, X_3 is an amino acid having an aromatic side chain, X_3 and X_4 are each any amino acid. In some embodiments, X is not a cysteine. In some embodiments, a synthetic IGF-II-R antigenic peptide is preferentially recognized by an IGF-II-R-specific CTL.

In some embodiments, synthetic IGF-II-R antigenic peptides bind with higher affinity to HLA-A2 molecules than does the corresponding native epitope, as shown in Table 2, Example 1.

Further provided herein are polypeptides comprising an amino acid sequence of a synthetic IGF-II-R antigenic peptide of the invention, including, but not limited to, FLFSWYAXV (SEQ ID NO:1); FLFSWFALV (SEQ ID NO:3);

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FLYSWWAIV (SEQ ID NO:5); FLYSWWWPV (SEQ ID NO:7); and/or FLFLWFFEV (SEQ ID NO:9), wherein X is any amino acid. In some embodiments, X is not a cysteine. Further provided are polypeptides comprising the consensus sequence $FLX_1X_2WX_3X_4X_5V$ (SEQ ID NO:13), wherein X_1 is F or Y or other amino acid, X_2 is S or L or other amino acid, X_3 is an amino acid having an aromatic side chain, X_3 and X_4 are each any amino acid. In some embodiments, X is not a cysteine. Further provided are polypeptides comprising one or more of these sequences wherein the polypeptides are preferentially recognized by IGF-II-R-specific cytotoxic T lymphocytes.

This invention also provides synthetic gp100 peptides having one or more of the following sequences, which are shown in FLDQVAFXV (SEQ ID NO:15); FLDQVAFVV (SEQ ID NO:17); FLFSWYAXV (SEQ ID NO:19); FLDQVPFSV (SEQ ID NO:21); FLDQRVFFV (SEQ ID NO:23); and/or FLDQRVFVV (SEQ ID NO:25), wherein X is any amino acid. In some embodiments, X is not cysteine. In a further embodiment, the present invention provides synthetic gp 100 peptides having the sequence XXDQXXFXX, (SEQ ID NO:37) wherein X is any amino acid, preferably a hydrophobic amino acid. In a further embodiment, X is not cysteine. Further provide are synthetic gp100 peptides having the sequence FLDQXXFXV (SEQ ID NO:27), wherein X is any amino acid. In some embodiments, X is not cysteine. Also encompassed by synthetic gp 100 peptides are peptides consisting essentially of one or more of the following sequences: FLDQVAFXV (SEQ ID NO:15); FLDQVAFVV (SEQ ID NO:17); FLFSWYAXV (SEQ ID NO:19); FLDQVPFSV (SEQ ID NO:21); FLDQRVFFV (SEQ ID NO:23); and/or FLDQRVFVV (SEQ ID NO:25), wherein X is any amino acid. In some embodiments, X is not cysteine. The peptides of the invention are particularly useful in that they have been shown to sensitize Hurley R1000 tumor infiltrating lymphocytes and to be potent antigens using the interferon-γ release assay (cytotoxic T cell specific for the G9-209 epitope encoded by the human melanoma associate protein gp100). The interferon-γ release assay, as well as assays for release of other cytokines from TILs, are known in the art and disclosed in Zugel et al. (1998) J. Immunol. **161**:1705-1709;

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Salgaller et al. (1995) *infra*; Rosenberg et al. (1998) Nature Med. **4**(3):321-327; Schwartzentruber et al. (1992) J. Immunol. **12**:1-12; and Schwartzentruber et al. (1994) J. Clin. Oncol. **12(7)**:1475-1483. The peptide sequences of the present invention differ from the natural epitope ITDQVPFSV (SEQ ID NO:29) in two ways: (1) they contain mutations in the putative HLA-A2 binding domain (amino acid residues 1, 2, and 9) conferring tighter binding to the MHC, and (2) they contain mutations in the putative T cell receptor-binding domain (amino acid residues 3-8) resulting in an apparent increased avidity for the T cell receptor. In one embodiment, the preferred amino acids are hydrophobic amino acids. The peptides also are novel over the synthetic peptides disclosed by Salgaller et al. (1995) Cancer Res. **55**:4972-4979.

The inventors determined that substituting positions 1 and 2 (Ile, Thr) of the native gp 100 epitope with Phe and Leu, respectively, while simultaneously maintaining the Val at position 9, resulted in higher binding affinity of a peptide epitope to HLA-A2, as shown below.

Thus, this invention also provides a method for enhancing the binding of a peptide epitope to HLA-A2 by substituting the amino acids at positions 1, 2, and 9, respectively, of the peptides sequence F, L, and V, respectively (SEQ ID NO:13).

Further encompassed by the term "synthetic gp100 peptide" are multimers (concatemers) of one or more of the foregoing sequences, optionally including intervening amino acid sequences. These multimers can be "homomultimers" or "heteromultimers," i.e., the multimers can comprise iterations of one of the above-listed sequences ("homomultimer"), or can comprise combinations of two or more of the above-listed sequences ("heteromultimers").

In some embodiments, a synthetic gp100 peptide is preferentially recognized by a gp100-specific CTL.

Mimetics of synthetic antigenic peptides of the invention

The invention further provides substances which are mimetics of synthetic antigenic peptides of the invention. A mimetic of the present invention mimics a

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synthetic peptide of the invention, i.e., it can elicit a cellular immune response to a peptide of the invention, including an APC which has presented on its surface a peptide of the invention. A mimetic can be synthesized, or can be identified by screening known or randomly synthesized compounds.

A mimetic may be any chemical substance which exhibits the requisite epitope, and thus may be, for example, a simple or complex organic or inorganic molecule; a polypeptide; a polynucleotide; a carbohydrate; a lipid; a lipopolysaccharide; a lipoprotein, or any combination of the above, including, but not limited to, a polynucleotide-containing polypeptide; a glycosylated polypeptide; and a glycolipid. A sample to be screened for the presence of a mimetic can be a biological sample, a library of randomly synthesized compounds, a library of known compounds, or any substance or mixture of substances.

By techniques well known in the art, mimetics can be designed based on the nucleic acid and amino acid sequences disclosed herein and the three-dimensional array or conformations of the amino acids, as determined by Fourier analysis of the X-ray diffraction patterns caused by crystals of polypeptide fragments bearing these amino acid sequences. U.S. Patent No. 5,648,379; Colman (1994) Protein Science 3:1687-1696; Malby et al. (1994) Structure 2:733-746; McCoy et al. (1997) J. Mol. Biol. **268**:570-584. By techniques well known in the art, mimetics may be designed based on the nucleic acid and amino acid sequences disclosed herein and the three-dimensional array or conformations of the amino acids, as determined by ^{1}H nuclear magnetic resonance (^{1}H NMR)-derived distance and dihedral angle constraints using distance geometry calculations, restrained simulated annealing, and conjugate gradient energy minimization measurements on the polypeptide fragments bearing these amino acid sequences, in various solvents. Pallaghy et al. (1995) Biochemistry **34**:3782-3794.

Substances which are mimetics of a synthetic antigenic peptide of the invention can also be screened from biological samples, libraries of known compounds and libraries of randomly- or non-randomly-generated synthetic compounds.

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Accordingly, the present invention provides methods (i.e., screening methods) of detecting (or identifying) compounds or substances which mimic a synthetic antigenic peptide of the invention (i.e., antigenic peptide mimetics). The methods generally involve contacting a sample to be tested for the presence of an antigenic peptide mimetic with an antibody specific for an antigenic peptide of the invention under suitable conditions and for a suitable time so as to allow specific binding (if any) to occur; and detecting specific binding. Conditions generally suitable for specific binding occur between about 4°C and about 40°C, preferably between about 4°C and about 37°C, in a buffered solution, and within a pH range of between 5 and 9. A variety of buffered solutions are known in the art, can be used in the screening methods of this invention, and include, but are not limited to, phosphate-buffered saline. Sufficient time for the binding and response will generally be between about 1 second and about 24 hours after exposure of the sample to the antibody. The mimetic can then be further characterized by testing for ability to elicit an immune effector cell response to an antigenic peptide of the invention when presented on an APC (or antigen-presenting matrix).

Detection of specific binding of a mimetic to an anti-synthetic antigen peptide antibody may be conducted using standard techniques in the art, such as ELISA, FACS, and competition assays. Such assays are well known in the art.

Determination of whether an analog (mimetic) of a synthetic antigenic peptide of the invention exhibits one or more of the functions of a synthetic antigenic peptide of the invention is well within the ability of those of ordinary skill in the art. For example, an assay to detect an immune effector cell specific which can lyse an APC presenting an antigenic peptide of the invention can be performed as described elsewhere herein.

The present invention also provides compositions, including pharmaceutical compositions, containing an antigenic peptide mimetic of the invention. Pharmaceutical excipitent suitable for use in a composition comprising a mimetic of the invention are known in the art and are set forth in REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition, Mack Publishing (1990). Other

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compositions comprise the mimetic in an appropriate solvent or solution, such as a buffer system. Such solvent systems are well known in the art.

Polynucleotides comprising sequences encoding antigenic peptides of the invention

The invention further provides isolated polynucleotides encoding synthetic antigenic peptides (and polypeptides) of the invention.

In some embodiments, the invention provides polynucleotides encoding synthetic IGF-II-R peptides having, or consisting essentially of, or polypeptides comprising, the sequences FLFSWYAXV (SEQ ID NO:1); FLFSWFALV (SEQ ID NO:3); FLYSWWAIV (SEQ ID NO:5); FLYSWWWPV (SEQ ID NO:7); and/or FLFLWFFEV (SEQ ID NO:9), wherein X is any amino acid, and the complements of these polynucleotides. In additional embodiments, X is not cysteine.

This invention also provides polynucleotides encoding synthetic gp100 peptides having one or more of the following sequences, which are shown in FLDQVAFXV (SEQ ID NO:15); FLDQVAFVV (SEQ ID NO:17); FLFSWYAXV (SEQ ID NO:19); FLDQVPFSV (SEQ ID NO:21); FLDQRVFFV (SEQ ID NO:23); and/or FLDQRVFVV (SEQ ID NO:25), wherein X is any amino acid. In some embodiments, X is not cysteine. In a further embodiment, the present invention provides synthetic gp 100 peptides having the sequence XXDQXXFXX, (SEQ ID NO:37) wherein X is any amino acid, preferably a hydrophobic amino acid. In a further embodiment, X is not cysteine. Further provide are synthetic gp100 peptides having the sequence FLDQXXFXV (SEQ ID NO:27), wherein X is any amino acid. In some embodiments, X is not cysteine. Also encompassed by synthetic gp 100 peptides are peptides consisting essentially of one or more of the following sequences: FLDQVAFXV (SEQ ID NO:15); FLDQVAFVV (SEQ ID NO:17); FLFSWYAXV (SEQ ID NO:19); FLDQVPFSV (SEQ ID NO:21); FLDQRVFFV (SEQ ID NO:23); and/or FLDQRVFVV (SEQ ID NO:25), wherein X is any amino acid. In some embodiments, X is not cysteine.

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As used herein, the term "polynucleotide" encompasses DNA, RNA and nucleic acid mimetics. In addition to the polynucleotide sequences encoding a synthetic antigenic peptide of the invention, or their complements, this invention also provides the anti-sense polynucleotide strand, e.g. antisense RNA to these sequences or their complements. Accordingly, in some embodiments, the invention provides polynucleotide sequences shown in SEQ ID NOS:2, 4, 6, 8, 10, 16, 18, 20, 22, 24 26 and 28, their complements, and corresponding anti-sense strands. One can obtain an antisense RNA using known sequences and the methodology described in Vander Krol et al. (1988) BioTechniques 6:958.

The polynucleotides can be conjugated to a detectable marker, e.g., an enzymatic label or a radioisotope for detection of nucleic acid and/or expression of the gene in a cell. A wide variety of appropriate detectable markers are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples. Briefly, this invention further provides a method for detecting a single-stranded polynucleotide or its complement, by contacting target single-stranded polynucleotides with a labeled, single-stranded polynucleotide (a probe) which is at least 4, and more preferably at least 5 or 6 and most preferably at least 10 of the 10 nucleotides of a polynucleotide of the invention (or the corresponding complement) under conditions permitting hybridization (preferably moderately stringent hybridization conditions) of complementary single-stranded polynucleotides, or more preferably, under highly stringent hybridization conditions. Hybridized polynucleotide pairs are separated from un-hybridized, single-stranded polynucleotides. The hybridized polynucleotide pairs are detected using methods

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well known to those of skill in the art and set forth, for example, in Sambrook et al. (1989) *supra*.

The polynucleotides of this invention can be replicated using PCR. PCR technology is the subject matter of United States Patent Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202 and described in PCR: THE POLYMERASE CHAIN REACTION (Mullis et al. eds., Birkhauser Press, Boston (1994)) and references cited therein.

Alternatively, one of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to replicate the DNA. Accordingly, this invention also provides a process for obtaining the polynucleotides of this invention by providing the linear sequence of the polynucleotide, appropriate primer molecules, chemicals such as enzymes and instructions for their replication and chemically replicating or linking the nucleotides in the proper orientation to obtain the polynucleotides. In a separate embodiment, these polynucleotides are further isolated. Still further, one of skill in the art can insert the polynucleotide into a suitable replication vector and insert the vector into a suitable host cell (prokaryotic or eukaryotic) for replication and amplification. The DNA so amplified can be isolated from the cell by methods well known to those of skill in the art. A process for obtaining polynucleotides by this method is further provided herein as well as the polynucleotides so obtained.

RNA can be obtained by first inserting a DNA polynucleotide into a suitable host cell. The DNA can be inserted by any appropriate method, e.g., by the use of an appropriate gene delivery vehicle (e.g., liposome, plasmid or vector) or by electroporation. When the cell replicates and the DNA is transcribed into RNA; the RNA can then be isolated using methods well known to those of skill in the art, for example, as set forth in Sambrook et al. (1989) *supra*. For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook et al. (1989) *supra* or extracted by nucleic-acid-binding resins following the accompanying instructions provided by manufactures.

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Polynucleotides having at least 4 contiguous nucleotides, and more preferably at least 5 or 6 contiguous nucleotides and most preferably at least 10 contiguous nucleotides, and exhibiting sequence complementarity or homology to in SEQ ID NOS:2, 4, 6, 8, 10, 16, 18, 20, 22, 24, 26, 28 and 37 find utility as hybridization probes.

It is known in the art that a "perfectly matched" probe is not needed for a specific hybridization. Minor changes in probe sequence achieved by substitution, deletion or insertion of a small number of bases do not affect the hybridization specificity. In general, as much as 20% base-pair mismatch (when optimally aligned) can be tolerated. Preferably, a probe useful for detecting the aforementioned mRNA is at least about 80% identical to the homologous region of comparable size contained in the previously identified sequences identified by in SEQ ID NOS:2, 4, 6, 8, 10, 16, 18, 20, 22, 24, 26 and 37, which correspond to previously characterized genes or in SEQ ID NOS:2, 4, 6, 8, 10, 16, 18, 20, 22, 24, 26 and 37. More preferably, the probe is 85% identical to the corresponding gene sequence after alignment of the homologous region; even more preferably, it exhibits 90% identity.

These probes can be used in radioassays (*e.g.* Southern and Northern blot analysis) to detect or monitor various cells or tissue containing these cells. The probes also can be attached to a solid support or an array such as a chip for use in high throughput screening assays for the detection of expression of the gene corresponding to one or more polynucleotide(s) of this invention. Accordingly, this invention also provides at least one probe as defined above of the transcripts identified as SEQ ID NOS: 2, 4, 6, 8, 10, 16, 18, 20, 22, 24, 26 and 37, or the complement of one of these sequences, attached to a solid support for use in high throughput screens.

The polynucleotides of the present invention also can serve as primers for the detection of genes or gene transcripts that are expressed in APC, for example, to confirm transduction of the polynucleotides into host cells. In this context, amplification means any method employing a primer-dependent polymerase capable of replicating a target sequence with reasonable fidelity. Amplification

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may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of *E. coli* DNA polymerase, and reverse transcriptase. A preferred length of the primer is the same as that identified for probes, above.

The invention further provides the isolated polynucleotide operatively linked to a promoter of RNA transcription, as well as other regulatory sequences for replication and/or transient or stable expression of the DNA or RNA. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct transcription of RNA off the DNA molecule. Examples of such promoters are SP6, T4 and T7. In certain embodiments, cell-specific promoters are used for cell-specific expression of the inserted polynucleotide. Vectors which contain a promoter or a promoter/enhancer, with termination codons and selectable marker sequences, as well as a cloning site into which an inserted piece of DNA can be operatively linked to that promoter are well known in the art and commercially available. For general methodology and cloning strategies, see GENE EXPRESSION TECHNOLOGY (Goeddel ed., Academic Press, Inc. (1991)) and references cited therein and VECTORS: ESSENTIAL DATA SERIES (Gacesa and Ramji, eds., John Wiley & Sons, N.Y. (1994)), which contains maps, functional properties, commercial suppliers and a reference to GenEMBL accession numbers for various suitable vectors. Preferably, these vectors are capable of transcribing RNA *in vitro* or *in vivo*.

Delivery vehicles comprising a polynucleotide of the invention

The present invention also provides delivery vehicles suitable for delivery of a polynucleotide of the invention into cells (whether *in vivo*, *ex vivo*, or *in vitro*). A polynucleotide of the invention can be contained within a cloning or expression vector. These vectors (especially expression vectors) can in turn be manipulated to assume any of a number of forms which may, for example, facilitate delivery to and/or entry into a cell.

Expression vectors containing these nucleic acids are useful to obtain host vector systems to produce proteins and polypeptides. It is implied that these

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expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include plasmids, viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, etc. Adenoviral vectors are particularly useful for introducing genes into tissues *in vivo* because of their high levels of expression and efficient transformation of cells both *in vitro* and *in vivo*. When a nucleic acid is inserted into a suitable host cell, e.g., a prokaryotic or a eukaryotic cell and the host cell replicates, the protein can be recombinantly produced. Suitable host cells will depend on the vector and can include mammalian cells, animal cells, human cells, simian cells, insect cells, yeast cells, and bacterial cells constructed using well known methods. See Sambrook, et al. (1989) *supra*. In addition to the use of viral vector for insertion of exogenous nucleic acid into cells, the nucleic acid can be inserted into the host cell by methods well known in the art such as transformation for bacterial cells; transfection using calcium phosphate precipitation for mammalian cells; or DEAE-dextran; electroporation; or microinjection. See Sambrook et al. (1989) *supra* for this methodology. Thus, this invention also provides a host cell, e.g., a mammalian cell, an animal cell (rat or mouse), a human cell, or a prokaryotic cell such as a bacterial cell, containing a polynucleotide encoding a protein or polypeptide or antibody.

When the vectors are used for gene therapy *in vivo* or *ex vivo*, a pharmaceutically acceptable vector is preferred, such as a replication-incompetent retroviral or adenoviral vector. Pharmaceutically acceptable vectors containing the nucleic acids of this invention can be further modified for transient or stable expression of the inserted polynucleotide. As used herein, the term "pharmaceutically acceptable vector" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the nucleic acid into dividing cells. An example of such a vector is a "replication-incompetent" vector defined by its inability to produce viral proteins, precluding spread of the vector in the infected host cell. An example of a replication-incompetent retroviral vector is LNL6. Miller et al. (1989) BioTechniques 7:980-990. The methodology of using replication-incompetent retroviruses for

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retroviral-mediated gene transfer of gene markers is well established. Correll et al. (1989) Proc. Natl. Acad. Sci. USA **86**:8912; Bordignon (1989) Proc. Natl. Acad. Sci. USA **86**:8912-52; Culver (1991) Proc. Natl. Acad. Sci. USA **88**:3155; and Rill (1991) Blood **79**(10):2694-700.

In general, genetic modifications of cells employed in the present invention are accomplished by introducing a vector containing a polynucleotide comprising sequences encoding a synthetic antigenic peptide of the invention. A variety of different gene transfer vectors, including viral as well as non-viral systems can be used.

A wide variety of non-viral vehicles for delivery of a polynucleotide of the invention are known in the art and are encompassed in the present invention. A polynucleotide of the invention can be delivered to a cell as naked DNA. WO 97/40163. Alternatively, a polynucleotide of the invention can be delivered to a cell associated in a variety of ways with a variety of substances (forms of delivery) including, but not limited to cationic lipids; biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria. A delivery vehicle may take the form of a microparticle. Mixtures or conjugates of these various substances can also be used as delivery vehicles. A polynucleotide of the invention can be associated with these various forms of delivery non-covalently or covalently.

Included in the non-viral vector category are prokaryotic plasmids and eukaryotic plasmids. Non-viral vectors (i.e., cloning and expression vectors) having cloned therein a polynucleotide(s) of the invention can be used for expression of recombinant polypeptides as well as a source of polynucleotide of the invention. Cloning vectors can be used to obtain replicate copies of the polynucleotides they contain, or as a means of storing the polynucleotides in a depository for future recovery. Expression vectors (and host cells containing these expression vectors) can be used to obtain polypeptides produced from the polynucleotides they contain. They may also be used where it is desirable to express polypeptides, encoded by an operably linked polynucleotide, in an

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individual, such as for eliciting an immune response via the polypeptide(s) encoded in the expression vector(s). Suitable cloning and expression vectors include any known in the art, *e.g.*, those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors and suitable host cells are known in the art and need not be described in detail herein. For example, see Gacesa and Ramji, Vectors, John Wiley & Sons (1994).

Cloning and expression vectors typically contain a selectable marker (for example, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the vector), although such a marker gene can be carried on another polynucleotide sequence co-introduced into the host cell. Only those host cells into which a selectable gene has been introduced will survive and/or grow under selective conditions. Typical selection genes encode protein(s) that (a) confer resistance to antibiotics or other toxins substances, *e.g.*, ampicillin, neomycyin, methotrexate, etc.; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from complex media. The choice of the proper marker gene will depend on the host cell, and appropriate genes for different hosts are known in the art. Cloning and expression vectors also typically contain a replication system recognized by the host.

Suitable cloning vectors may be constructed according to standard techniques, or may be selected from a large number of cloning vectors available in the art. While the cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, and/or may carry genes for a marker that can be used in selecting clones containing the vector. Suitable examples include plasmids and bacterial viruses, *e.g.*, pUC18, pUC19, Bluescript (*e.g.*, pBS SK+) and its derivatives, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28. These and many other cloning vectors are available from commercial vendors such as BioRad, Strategene, and Invitrogen. The Examples provided herein also provide examples of cloning vectors.

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Expression vectors generally are replicable polynucleotide constructs that contain a polynucleotide encoding a polypeptide of interest. The polynucleotide encoding the polypeptide of interest is operably linked to suitable transcriptional controlling elements, such as promoters, enhancers and terminators. For expression (i.e., translation), one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites, and stop codons. A polynucleotide sequence encoding a signal peptide can also be included to allow a polypeptide, encoded by an operably linked polynucleotide, to cross and/or lodge in cell membranes or be secreted from the cell. A number of expression vectors suitable for expression in eukaryotic cells including yeast, avian, and mammalian cells are known in the art. Examples of mammalian expression vectors contain both prokaryotic sequence to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. Examples of mammalian expression vectors suitable for transfection of eukaryotic cells include the pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pRSVneo, and pHyg derived vectors. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEB, pREP derived vectors) can be used for expression in mammalian cells. Examples of expression vectors for yeast systems, include YEP24, YIP5, YEP51, YEP52, YES2 and YRP17, which are cloning and expression vehicles useful for introduction of constructs into S. cerevisiae. Broach et al. (1983) *Experimental Manipulation of Gene Expression*, ed. M. Inouye, Academic Press. p. 83. Baculovirus expression vectors for expression in insect cells include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors and pBlueBac-derived vectors.

Viral vectors include, but are not limited to, DNA viral vectors such as those based on adenoviruses, herpes simplex virus, poxviruses such as vaccinia virus, and parvoviruses, including adeno-associated virus; and RNA viral vectors, including, but not limited to, the retroviral vectors. Retroviral vectors include murine leukemia virus, and lentiviruses such as human immunodeficiency virus. Naldini et al. (1996) Science 272:263-267.

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Replication-defective retroviral vectors harboring a polynucleotide of the invention as part of the retroviral genome can be used. Such vectors have been described in detail. (Miller et al. (1990) Mol. Cell Biol. **10**:4239; Kolberg, R. (1992) J. NIH Res. **4**:43; Cornetta et al. (1991) Hum. Gene Ther **2**:215).

Adenovirus and adeno-associated virus vectors useful in the genetic modifications of this invention may be produced according to methods already taught in the art. (See, e.g., Karlsson et al. (1986) EMBO 5:2377; Carter (1992) Current Opinion in Biotechnology 3:533-539; Muzcyzka (1992) Current Top. Microbiol. Immunol. **158**:97-129; GENE TARGETING: A PRACTICAL APPROACH (1992) ed. A. L. Joyner, Oxford University Press, NY). Several different approaches are feasible.

Additional references describing viral vectors which could be used in the methods of the present invention include the following: Horwitz, M.S., Adenoviridae and Their Replication, in Fields, B., et al. (eds.) VIROLOGY, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham, F. et al., pp. 109-128 in METHODS IN MOLECULAR BIOLOGY, Vol. 7: GENE TRANSFER AND EXPRESSION PROTOCOLS, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller et al. (1995) FASEB Journal **9**:190-199, Schreier (1994) Pharmaceutica Acta Helvetiae **68**:145-159; Schneider and French (1993) Circulation **88**:1937-1942; Curiel et al. (1992) Human Gene Therapy **3**:147-154; Graham et al. WO 95/00655 (5 January 1995); Falck-Pedersen WO 95/16772 (22 June 1995); Denefle et al. WO 95/23867 (8 September 1995); Haddada et al. WO 94/26914 (24 November 1994); Perricaudet et al. WO 95/02697 (26 January 1995); and Zhang et al. WO 95/25071 (12 October 1995).

The efficiency of transduction of DCs or other APCs can be assessed by immunofluorescence using fluorescent antibodies specific for the tumor antigen being expressed (Kim et al. (1997) J. Immunother. **20**:276-286). Alternatively, the antibodies can be conjugated to an enzyme (e.g. HRP) giving rise to a colored product upon reaction with the substrate. The actual amount of antigenic polypeptides being expressed by the APCs can be evaluated by ELISA.

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In vivo transduction of DCs, or other APCs, can be accomplished by administration of a viral vectors comprising a polynucleotide of the invention via different routes including intravenous, intramuscular, intranasal, intraperitoneal or cutaneous delivery. One method which can be used is cutaneous delivery of Ad vector at multiple sites using a total dose of approximately $1x10^{10}$-$1x\ 10^{12}$ i.u. Levels of *in vivo* transduction can be roughly assessed by co-staining with antibodies directed against APC marker(s) and the peptide epitope being expressed. The staining procedure can be carried out on biopsy samples from the site of administration or on cells from draining lymph nodes or other organs where APCs (in particular DCs) may have migrated. Condon et al. (1996) Nature Med. **2**:1122-1128; Wan et al. (1997) Human Gene Therapy **8**:1355-1363. The amount of antigen being expressed at the site of injection or in other organs where transduced APCs may have migrated can be evaluated by ELISA on tissue homogenates.

APCs can also be transduced *in vitro/ex vivo* by non-viral gene delivery methods such as electroporation, calcium phosphate precipitation or cationic lipid/plasmid DNA complexes. Arthur et al. (1997) Cancer Gene Therapy **4**:17-25. Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

In vivo transduction of DCs, or other APCs, can potentially be accomplished by administration of cationic lipid/plasmid DNA complexes delivered via the intravenous, intramuscular, intranasal, intraperitoneal or cutaneous route of administration. Gene gun delivery or injection of naked plasmid DNA into the skin also leads to transduction of DCs. Condon et al. (1996) Nature Med. **2**:1122-1128; Raz et al. (1994) Proc. Natl. Acad. Sci. USA **91**:9519-9523. Intramuscular delivery of plasmid DNA may also be used for immunization. Rosato et al. (1997) Human Gene Therapy **8**:1451-1458.

The transduction efficiency and levels of transgene expression can be assessed as described above for viral vectors.

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Databases of the sequences of polynucleotides of the invention and high throughput screens

The sequences of polynucleotides of this invention also can be used for comparison to known and unknown sequences using a computer-based method to match a sample sequence with known sequences. Thus, this invention also provides the sequences of the polynucleotides of this invention in a computer database or in computer readable form, including applications utilizing the internet.

A linear search through such a database may be used. Alternatively, the polynucleotide sequence can be converted into a unique numeric representation. The comparison aspects may be implemented in hardware or software, or a combination of both. Preferably, these aspects of the invention are implemented in computer programs executing on a programable computer comprising a processor, a data storage system (including volatile and non-volatile memory and/or storage elements), at least one input device, and at least one output device. Data input through one or more input devices for temporary or permanent storage in the data storage system includes sequences, and may include previously generated polynucleotides and codes for known and/or unknown sequences. Program code is applied to the input data to perform the functions described above and generate output information. The output information is applied to one or more output devices, in known fashion.

Each such computer program is preferably stored on a storage media or device (*e.g.*, ROM or magnetic diskette) readable by a general or special purpose programmable computer, for configuring and operating the computer when the storage media or device is read by the computer to perform the procedures described herein. The inventive system may also be considered to be implemented as a computer-readable storage medium, configured with a computer program, where the storage medium so configured causes a computer to operate in a specific and predefined manner to perform the functions described herein.

A polynucleotide of the invention also can be attached to a solid support for use in high throughput screening assays. PCT WO 97/10365, for example,

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discloses the construction of high density oligonucleotide chips. See also, U.S. Patent Nos. 5,405,783; 5,412,087; and 5,445,934. Using this method, the probes are synthesized on a derivatized glass surface. Photoprotected nucleoside phosphoramidites are coupled to the glass surface, selectively deprotected by photolysis through a photolithographic mask, and reacted with a second protected nucleoside phosphoramidite. The coupling/deprotection process is repeated until the desired probe is complete.

The expression level of a gene is determined through exposure of a nucleic acid sample to the probe-modified chip. Extracted nucleic acid is labeled, for example, with a fluorescent tag, preferably during an amplification step. Hybridization of the labeled sample is performed at an appropriate stringency level. The degree of probe-nucleic acid hybridization is quantitatively measured using a detection device, such as a confocal microscope. See U.S. Patent Nos. 5,578,832; and 5,631,734. The obtained measurement is directly correlated with gene expression level.

Results from the chip assay are typically analyzed using a computer software program. See, for example, EP 717,113 A2 and WO 95/20681. The hybridization data is read into the program, which calculates the expression level of the targeted gene(s). This figure is compared against existing data sets of gene expression levels for that cell type.

For example, the database and methods of using the database provides a means to differentiate a cell expressing a peptide epitope which is the natural counterpart of a synthetic antigenic peptide epitope of the invention from a cell which does not express the epitope or expresses it at a higher or lower level from the cell in question. Expression of polynucleotides encoding the peptide is measured. One cell would serve as a "reference cell" and the cell whose expression of a polynucleotide encoding a peptide corresponding to a synthetic antigenic peptide epitope of the invention is to be measured could be referred to as the "test cell." As an example, the method can be used to distinguish a normal cell (in this case, the reference cell) from a neoplastic cell (i.e., the test cell). It also allows one to differentiate between neoplastic cells biopsied from different

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regions from a patient or different subjects or gene expression before or after treatment with a potential therapeutic agent. It can be used to analyze drug toxicity and efficacy, as well as to selectively look at protein categories which are expected to be affected by a drug or which may be overexpressed as a result of treatment with a drug, such as the various multi-drug resistant genes. Additional utilities of the database include, but are not limited to analysis of the developmental state of a test cell, the influence of viral or bacterial infection, control of cell cycle, effect of a tumor suppressor gene or lack thereof, polymorphism within the cell type, apoptosis, and the effect of regulatory genes.

Host cells comprising polynucleotides of the invention

The present invention further provides host cells comprising polynucleotides of the invention. Host cells containing the polynucleotides of this invention are useful for the recombinant replication of the polynucleotides and for the recombinant production of peptides of the invention. Alternatively, host cells comprising a polynucleotide of the invention may be used to induce an immune response in a subject in the methods described herein.

Host cells which are suitable for recombinant replication of the polynucleotides of the invention, and for the recombinant production of peptides of the invention can be prokaryotic or eukaryotic. Host systems are known in the art and need not be described in detail herein. Prokaryotic hosts include bacterial cells, for example *E. coli*, *B. subtilis*, and mycobacteria. Among eukaryotic hosts are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian cells. These cells are cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

When the host cells are antigen presenting cells, they can be used to expand a population of immune effector cells such as tumor infiltrating lymphocytes which in turn are useful in adoptive immunotherapies. Antigen presenting cells are described in more detail below.

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Antibodies which bind specifically to peptides of the invention

Also provided by this invention is an antibody capable of specifically forming a complex with a peptide(s) and/or polypeptide(s) of this invention. The term "antibody" includes polyclonal antibodies and monoclonal antibodies. The antibodies include, but are not limited to mouse, rat, and rabbit or human antibodies. The antibodies are useful to identify and purify peptides/polypeptides of the invention and APCs expressing the peptides/polypeptides.

Laboratory methods for producing polyclonal antibodies and monoclonal antibodies, as well as deducing their corresponding nucleic acid sequences, are known in the art, see Harlow and Lane (1988) *Supra* and Sambrook et al. (1989) *Supra*. The monoclonal antibodies of this invention can be biologically produced by introducing protein or a fragment thereof into an animal, e.g., a mouse or a rabbit. The antibody producing cells in the animal are isolated and fused with myeloma cells or heteromyeloma cells to produce hybrid cells or hybridomas. Accordingly, the hybridoma cells producing the monoclonal antibodies of this invention also are provided.

Thus, using the protein or fragment thereof, and well known methods, one of skill in the art can produce and screen the hybridoma cells and antibodies of this invention for antibodies having the ability to bind the proteins or polypeptides.

If a monoclonal antibody being tested binds with the peptide or polypeptide, then the antibody being tested and the antibodies provided by the hybridomas of this invention are equivalent. It also is possible to determine without undue experimentation, whether an antibody has the same specificity as the monoclonal antibody of this invention by determining whether the antibody being tested prevents a monoclonal antibody of this invention from binding the peptide or polypeptide with which the monoclonal antibody is normally reactive. If the antibody being tested competes with the monoclonal antibody of the invention as shown by a decrease in binding by the monoclonal antibody of this invention, then it is likely that the two antibodies bind to the same or a closely

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related epitope. Alternatively, one can pre-incubate the monoclonal antibody of this invention with a protein with which it is normally reactive, and determine if the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of this invention.

The term "antibody" also is intended to include antibodies of all isotypes. Particular isotypes of a monoclonal antibody can be prepared either directly by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class switch variants using the procedure described in Steplewski et al. (1985) Proc. Natl. Acad. Sci. USA **82**:8653 or Spira et al. (1984) J. Immunol. Methods **74**:307.

This invention also provides biological active fragments of the polyclonal and monoclonal antibodies described above. These "antibody fragments" retain some ability to selectively bind with its antigen or immunogen. Such antibody fragments can include, but are not limited to:

(1) Fab,

(2) Fab',

(3) $F(ab')_2$,

(4) Fv, and

(5) SCA (single chain antibody)

A specific example of "a biologically active antibody fragment" is a CDR region of the antibody. Methods of making these fragments are known in the art, see for example, Harlow and Lane (1988) *Supra*.

The antibodies of this invention also can be modified to create chimeric antibodies and humanized antibodies (Oi et al. (1986) BioTechniques **4**(3):214). Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species.

The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the invention can also be

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accomplished by one of ordinary skill in the art by producing anti-idiotypic antibodies. Herlyn et al. (1986) Science 232:100. An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the monoclonal antibody produced by the hybridoma of interest.

Idiotypic identity between monoclonal antibodies of two hybridomas suggests that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using antibodies to the epitopic determinants on a monoclonal antibody it is possible to identify other hybridomas expressing monoclonal antibodies of the same epitopic specificity.

It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody can have a binding domain in the hypervariable region which is the mirror image of the epitope bound by the first monoclonal antibody. Thus, in this instance, the anti-idiotypic monoclonal antibody could be used for immunization for production of these antibodies.

"Epitope" refers to that portion of a molecule which is specifically recognized
by an antibody or a T cell antigen receptor. It is also referred to as an "antigenic determinant" or an "antigenic region." Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The antibodies of this invention can be linked to a detectable agent or label. There are many different labels and methods of labeling known to those of ordinary skill in the art.

The coupling of antibodies to low molecular weight haptens can increase the sensitivity of the assay. The haptens can then be specifically detected by means of a second reaction. For example, it is common to use haptens such as biotin (which reacts with avidin), or dinitrophenyl, pyridoxal, and fluorescein,

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which can react with specific anti-hapten antibodies. See Harlow and Lane (1988) *Supra*.

The monoclonal antibodies of the invention also can be bound to many different carriers. Thus, this invention also provides compositions containing the antibodies and another substance, active or inert. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

Compositions containing the antibodies, fragments thereof or cell lines which produce the antibodies, are encompassed by this invention. When these compositions are to be used pharmaceutically, they are combined with a pharmaceutically acceptable carrier.

Host cells presenting synthetic antigenic peptides of the invention

The invention further provides isolated host cells comprising synthetic antigenic peptides of the invention. In some embodiments, these host cells present one or more peptides of the invention on the surface of the cell in the context of an MHC molecule, i.e., a synthetic antigenic peptide of the invention is bound to a cell surface MHC molecule such that the peptide can be recognized by an immune effector cell. Isolated host cells which present the polypeptides of this invention in the context of MHC molecules are further useful to expand and isolate a population of educated, antigen-specific immune effector cells. The immune effector cells, *e.g.*, cytotoxic T lymphocytes, are produced by culturing naïve immune effector cells with antigen-presenting cells cells which present the polypeptides in the context of MHC molecules on the surface of the APCs. The population can be purified using methods known in the art, *e.g.*, FACS analysis or FICOLL™ gradient. The methods to generate and culture the immune effector cells as well as the populations produced thereby also are the inventor's

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contribution and invention. Pharmaceutical compositions comprising the cells and pharmaceutically acceptable carriers are useful in adoptive immunotherapy. Prior to administration *in vivo*, the immune effector cells are screened *in vitro* for their ability to lyse melanoma tumor cells.

In some of these embodiments, isolated host cells are APCs. APCs include, but are not limited to, dendritic cells (DCs), monocytes/macrophages, B lymphocytes or other cell type(s) expressing the necessary MHC/co-stimulatory molecules.

In some embodiments, the immune effector cells and/or the APCs are genetically modified. Using standard gene transfer, genes coding for co-stimulatory molecules and/or stimulatory cytokines can be inserted prior to, concurrent to or subsequent to expansion of the immune effector cells.

Antigen-presenting matrices comprising peptides of the invention

A synthetic antigenic epitope of the invention can be presented (bound by) an MHC Class I or Class II molecule in an antigen-presenting matrix, with or without co-stimulatory molecules necessary for CD4+ or CD8+ T cell activation. Whether co-stimulatory molecules are present may depend on the intended use of the antigen-presenting matrix.

Antigen-presenting matrices include those on the surface of an APC as well as synthetic antigen-presenting matrices. Antigen-presenting matrices are a form of solid support. APCs suitable for use in the present invention are capable of presenting exogenous peptide or protein or endogenous antigen to T cells in association with an antigen-presenting molecule, such as an MHC molecule. APCs include, but are not limited to, macrophages, dendritic cells, CD40-activated B cells, antigen-specific B cells, tumor cells, virus-infected cells, and genetically modified cells.

APCs can obtained from a variety of sources, including but not limited to, peripheral blood mononuclear cells (PBMC), whole blood or fractions thereof containing mixed populations, spleen cells, bone marrow cells, tumor infiltrating lymphocytes, cells obtained by leukapheresis, lymph nodes, e.g., lymph nodes

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draining from a tumor. Suitable donors include an immunized donor, a non-immunized (naïve) donor, treated or untreated donors. A "treated" donor is one that has been exposed to one or more biological modifiers. An "untreated" donor has not been exposed to one or more biological modifiers. APC's can also be treated *in vitro* with one or more biological modifiers.

The APCs are generally alive but can also be irradiated, mitomycin C treated, attenuated, or chemically fixed. Further, the APCs need not be whole cells. Instead, vesicle preparations of APCs can be used.

APCs can be genetically modified, i.e., transfected with a recombinant polynucleotide construct such that they express a polypeptide or an RNA molecule which they would not normally express or would normally express at lower levels. Examples of polynucleotides include, but are not limited to, those which encode an MHC molecule; a co-stimulatory molecule such as B7; and a peptide or polypeptide of the invention.

Cells which do not normally function *in vivo* in mammals as APCs can be modified in such a way that they function as APCs. A wide variety of cells can function as APCs when appropriately modified. Examples of such cells are insect cells, for example *Drosophila* or *Spodoptera*; and foster cells, such as the human cell line T2. For example, expression vectors which direct the synthesis of one or more antigen-presenting polypeptides, such as MHC molecules, optionally also accessory molecules such as B7, can be introduced into these cells to effect the expression on the surface of these cells antigen presentation molecules and, optionally, accessory molecules or functional portions thereof. Alternatively, antigen-presenting polypeptides and accessory molecules which can insert themselves into the cell membrane can be used. For example, glycosyl-phosphotidylinositol (GPI)-modified polypeptides can insert themselves into the membranes of cells. Hirose et al. (1995) Methods Enzymol. **250**:582-614; and Huang et al. (1994) Immunity **1**:607-613. Accessory molecules include, but are not limited to, co-stimulatory antibodies such as antibodies specific for CD28, CD80, or CD86; costimulatory molecules, including, but not limited to, B7.1 and B7.2; adhesion molecules such as ICAM-1 and LFA-3; and survival molecules

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such as Fas ligand and CD70. See, for example, PCT Publication No. WO 97/46256.

Foster antigen presenting cells are particularly useful as APCs. Foster APCs are derived from the human cell line 174xCEM.T2, referred to as T2, which contains a mutation in its antigen processing pathway that restricts the association of endogenous peptides with cell surface MHC class I molecules. Zweerink et al. (1993) J. Immunol. **150**:1763-1771. This is due to a large homozygous deletion in the MHC class II region encompassing the genes TAP1, TAP2, LMP1, and LMP2, which are required for antigen presentation to MHC class 1-restricted $CD8^+$ CTLs. In effect, only "empty" MHC class I molecules are presented on the surface of these cells. Exogenous peptide added to the culture medium binds to these MHC molecules provided that the peptide contains the allele-specific binding motif. These T2 cells are referred to herein as "foster" APCs. They can be used in conjunction with this invention to present antigen(s).

Transduction of T2 cells with specific recombinant MHC alleles allows for redirection of the MHC restriction profile. Libraries tailored to the recombinant allele will be preferentially presented by them because the anchor residues will prevent efficient binding to the endogenous allele.

High level expression of MHC molecules makes the APC more visible to the CTLs. Expressing the MHC allele of interest in T2 cells using a powerful transcriptional promoter (e.g., the CMV promoter) results in a more reactive APC (most likely due to a higher concentration of reactive MHC-peptide complexes on the cell surface).

Alternatively, a synthetic antigen-presenting matrix can be used to present antigen to an effector cell(s). A synthetic matrix can include an antigen presenting molecule, preferably an MHC Class I or MHC Class II molecule, immobilized on a solid support, for example, beads or plates. Accessory molecules can be present, which can be co-immobilized or soluble, the molecules including, but not limited to, co-stimulatory antibodies such as antibodies specific for CD28, CD80, or CD86; costimulatory molecules, including, but not limited to, B7.1 and B7.2; adhesion molecules such as ICAM-1 and LFA-3; and survival molecules such as

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Fas ligand and CD70. Portions of accessory molecules can also be used, as long as their function is maintained. Solid supports include metals or plastics, porous materials, microbeads, microtiter plates, red blood cells, and liposomes. See, for example, International Patent Publication Nos. WO 97/46256; and WO 97/35035.

Methods for determining whether an antigen-presenting matrix, whether it is on a cell surface or on a synthetic support, is capable of presenting antigen to an immune effector cell in such a manner as to effect activation of the immune effector cell, are known in the art and include, for example, ^{3}H-thymidine uptake by effector cells, cytokine production by effector cells, and cytolytic ^{51}Cr-release assays.

In some embodiments, a synthetic antigenic peptide of the invention is presented on an antigen-presenting matrix in a Class I or Class II MHC molecule such that the peptide is bound by a TCR on a CD4+ or CD8+ T cell, but the antigen-presenting matrix lacks one or more co-stimulatory molecules required for activation of the T cell. These antigen-presenting matrices induce T cell anergy (unresponsiveness), and are useful in methods described herein for reducing or suppressing an immune response. Methods for determining whether an antigen-presenting matrix is capable of presenting antigen to an immune effector cell, in such a manner as to effect T cell anergy, are known in the art.

The following is a brief description of two fundamental approaches for the isolation of APC. These approaches involve (1) isolating bone marrow precursor cells ($CD34^{+}$) from blood and stimulating them to differentiate into APC; or (2) collecting the precommitted APCs from peripheral blood. In the first approach, the patient must be treated with cytokines such as GM-CSF to boost the number of circulating $CD34^{+}$ stem cells in the peripheral blood.

The second approach for isolating APCs is to collect the relatively large numbers of precommitted APCs already circulating in the blood. Previous techniques for isolating committed APCs from human peripheral blood have involved combinations of physical procedures such as metrizamide gradients and adherence/nonadherence steps (Freudenthal et al. (1990) Proc. Natl. Acad. Sci. USA 87:7698-7702); Percoll gradient separations (Mehta-Damani et al. (1994) J.

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Immunol. **153**:996-1003); and fluorescence activated cell sorting techniques (Thomas et al. (1993) J. Immunol. **151**:6840-52).

One technique for separating large numbers of cells from one another is known as countercurrent centrifugal elutriation (CCE). In this technique, cells are subject to simultaneous centrifugation and a washout stream of buffer which is constantly increasing in flow rate. The constantly increasing countercurrent flow of buffer leads to fractional cell separations that are largely based on cell size.

In one aspect of the invention, the APC are precommitted or mature dendritic cells which can be isolated from the white blood cell fraction of a mammal, such as a murine, simian or a human (See, e.g., WO 96/23060). The white blood cell fraction can be from the peripheral blood of the mammal. This method includes the following steps: (a) providing a white blood cell fraction obtained from a mammalian source by methods known in the art such as leukopheresis; (b) separating the white blood cell fraction of step (a) into four or more subfractions by countercurrent centrifugal elutriation, (c) stimulating conversion of monocytes in one or more fractions from step (b) to dendritic cells by contacting the cells with calcium ionophore, GM-CSF and IL-13 or GM-CSF and IL-4, (d) identifying the dendritic cell-enriched fraction from step (c), and (e) collecting the enriched fraction of step (d), preferably at about 4°C. One way to identify the dendritic cell-enriched fraction is by fluorescence-activated cell sorting. The white blood cell fraction can be treated with calcium ionophore in the presence of other cytokines, such as recombinant (rh) rhIL-12, rhGM-CSF, or rhIL-4. The cells of the white blood cell fraction can be washed in buffer and suspended in Ca^{++}/Mg^{++} free media prior to the separating step. The white blood cell fraction can be obtained by leukopheresis. The dendritic cells can be identified by the presence of at least one of the following markers: HLA-DR, HLA-DQ, or B7. 2, and the simultaneous absence of the following markers: CD3, CD14, CD16, 56, 57, and CD 19, 20. Monoclonal antibodies specific to these cell surface markers are commercially available.

More specifically, the method requires collecting an enriched collection of white cells and platelets from leukopheresis that is then further fractionated by

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countercurrent centrifugal elutriation (CCE). Abrahamsen et al. (1991) J. Clin. Apheresis 6:48-53. Cell samples are placed in a special elutriation rotor. The rotor is then spun at a constant speed of, for example, 3000 rpm. Once the rotor has reached the desired speed, pressurized air is used to control the flow rate of cells. Cells in the elutriator are subjected to simultaneous centrifugation and a washout stream of buffer which is constantly increasing in flow rate. This results in fractional cell separations based largely but not exclusively on differences in cell size.

Quality control of APC and more specifically DC collection and confirmation of their successful activation in culture is dependent upon a simultaneous multi-color FACS analysis technique which monitors both monocytes and the dendritic cell subpopulation as well as possible contaminant T lymphocytes. It is based upon the fact that DCs do not express the following markers: CD3 (T cell); CD14 (monocyte); CD16, 56, 57 (NK/LAK cells); CD19, 20 (B cells). At the same time, DCs do express large quantities of HLA-DR, significant HLA-DQ and B7.2 (but little or no B7.1) at the time they are circulating in the blood (in addition they express Leu M7 and M9, myeloid markers which are also expressed by monocytes and neutrophils).

Once collected, the DC rich/monocyte APC fractions (usually 150 through 190) can be pooled and cryopreserved for future use, or immediately placed in short term culture.

Alternatively, others have reported that a method for upregulating (activating) dendritic cells and converting monocytes to an activated dendritic cell phenotype. This method involves the addition of calcium ionophore to the culture media convert monocytes into activated dendritic cells. Adding the calcium ionophore A23187, for example, at the beginning of a 24-48 hour culture period resulted in uniform activation and dendritic cell phenotypic conversion of the pooled "monocyte plus DC" fractions: characteristically, the activated population becomes uniformly CD14 (Leu M3) negative, and upregulates HLA-DR, HLA-DQ, ICAM-1, B7.1, and B7.2.

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Specific combination(s) of cytokines have been used successfully to amplify (or partially substitute) for the activation/conversion achieved with calcium ionophore: these cytokines include but are not limited to purified or recombinant human ("rh") rhGM-CSF, rhIL-2, and rhIL-4. Each cytokine when given alone is inadequate for optimal upregulation.

Presentation of synthetic antigenic peptide epitopes of the invention by antigen-presenting matrices

For use in immunomodulatory methods and diagnostic methods of the invention, an antigen-presenting matrix presents a synthetic antigenic peptide epitope of the invention bound to an MHC molecule. Any known method can be used to achieve presentation of a synthetic antigenic peptide epitope of the invention by an antigen-presenting matrix. The following are non-limiting examples of methods which can be used.

A synthetic antigenic peptide epitope can be delivered to antigen-presenting cells as polypeptide or peptide or in the form of cDNA encoding the protein/peptide.

Another method to deliver a synthetic antigenic peptide epitope of the invention to an APC is by pulsing. Pulsing can be accomplished *in vitro/ex vivo* by exposing APCs to the antigenic polypeptide(s) or peptide(s) of this invention. The polypeptide(s) or peptide(s) are added to APCs at a concentration of 1-10 μm for approximately 3 hours. Pulsed APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

Synthetic antigenic peptide epitopes of the invention can also be delivered *in vivo*, for example, as part of a polypeptide or complexed with another macromolecule, with or without adjuvant via the intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

Various other techniques can be used, including the following. Paglia et al. (1996) J. Exp. Med. **183**:317-322 has shown that APC incubated with whole protein *in vitro* are recognized by MHC class I-restricted CTLs, and that

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immunization of animals with these APCs led to the development of antigen-specific CTLs *in vivo*. In addition, several different techniques have been described which lead to the expression of antigen in the cytosol of APCs, such as DCs. These include (1) the introduction into the APCs of RNA isolated from tumor cells, (2) infection of APCs with recombinant vectors to induce endogenous expression of antigen, and (3) introduction of tumor antigen into the DC cytosol using liposomes. (See Boczkowski et al. (1996) J. Exp. Med. **184**:465-472; Rouse et al. (1994) J. Virol. **68**:5685-5689; and Nair et al. (1992) J. Exp. Med. **175**:609-612).

Another method which can be used is termed "painting." It has been demonstrated that glycosyl-phosphotidylinositol (GPI)-modified proteins possess the ability to reincorporate themselves back into cell membranes after purification. Hirose et al. (1995) Methods Enzymol. **250**:582-614; Medof et al., (1984) J. Exp. Med. **160**:1558-1578; Medof (1996) FASEB J. **10**:574-586; and Huang et al. (1994) Immunity **1**:607-613 have exploited this property in order to create APCs of specific composition for the presentation of antigen to CTLs. They devised expression vectors for β2-microglobulin and the HLA-A2.1 allele. The proteins were expressed in Schneider S2 *Drosophila melanogaster* cells, known to support GPI-modification. After purification, the proteins could be incubated together with a purified antigenic peptide which resulted in a trimolecular complex capable of efficiently inserting itself into the membranes of autologous cells. In essence, these protein mixtures were used to "paint" the APC surface, conferring the ability to stimulate a CTL clone that was specific for the antigenic peptide. Cell coating was shown to occur rapidly and to be protein concentration dependent. This method of generating APCs bypasses the need for gene transfer into the APC and permits control of antigenic peptide densities at the cell surfaces.

Immune Effector Cells

The present invention makes use of the above-described antigen-presenting matrices, including APCs, to stimulate production of an enriched population of antigen-specific immune effector cells. Accordingly, the present invention

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provides a population of cells enriched in educated, antigen-specific immune effector cells, specific for an antigenic peptide of the invention. These cells can cross-react with (bind specifically to) antigenic determinants (epitopes) on natural (endogenous) antigens. In some embodiments, the natural antigen is on the surface of tumor cells and the educated, antigen-specific immune effector cells of the invention suppress growth of the tumor cells. When APCs are used, the antigen-specific immune effector cells are expanded at the expense of the APCs, which die in the culture. The process by which naïve immune effector cells become educated by other cells is described essentially in Coulie (1997) Molec. Med. Today 3:261-268.

The APCs prepared as described above are mixed with naïve immune effector cells. Preferably, the cells may be cultured in the presence of a cytokine, for example IL2. Because dendritic cells secrete potent immunostimulatory cytokines, such as IL-12, it may not be necessary to add supplemental cytokines during the first and successive rounds of expansion. In any event, the culture conditions are such that the antigen-specific immune effector cells expand (i.e. proliferate) at a much higher rate than the APCs. Multiple infusions of APCs and optional cytokines can be performed to further expand the population of antigen-specific cells.

In one embodiment, the immune effector cells are T cells. In a separate embodiment, the immune effector cells can be genetically modified by transduction with a transgene coding for example, IL-2, IL-11 or IL-13. Methods for introducing transgenes *in vitro*, *ex vivo* and *in vivo* are well known in the art. See Sambrook, et al. (1989) *Supra*.

An effector cell population suitable for use in the methods of the present invention can be autogeneic or allogeneic, preferably autogeneic. When effector cells are allogeneic, preferably the cells are depleted of alloreactive cells before use. This can be accomplished by any known means, including, for example, by mixing the allogeneic effector cells and a recipient cell population and incubating them for a suitable time, then depleting $CD69^+$ cells, or inactivating alloreactive cells, or inducing anergy in the alloreactive cell population.

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Hybrid immune effector cells can also be used. Immune effector cell hybrids are known in the art and have been described in various publications. See, for example, International Patent Application Nos. WO 98/46785; and WO 95/16775.

The effector cell population can comprise unseparated cells, i.e., a mixed population, for example, a PBMC population, whole blood, and the like. The effector cell population can be manipulated by positive selection based on expression of cell surface markers, negative selection based on expression of cell surface markers, stimulation with one or more antigens *in vitro* or *in vivo*, treatment with one or more biological modifiers *in vitro* or *in vivo*, subtractive stimulation with one or more antigens or biological modifiers, or a combination of any or all of these.

Effector cells can obtained from a variety of sources, including but not limited to, PBMC, whole blood or fractions thereof containing mixed populations, spleen cells, bone marrow cells, tumor infiltrating lymphocytes, cells obtained by leukapheresis, biopsy tissue, lymph nodes, e.g., lymph nodes draining from a tumor. Suitable donors include an immunized donor, a non-immunized (naïve) donor, treated or untreated donors. A "treated" donor is one that has been exposed to one or more biological modifiers. An "untreated" donor has not been exposed to one or more biological modifiers.

Methods of extracting and culturing effector cells are well known. For example, effector cells can be obtained by leukapheresis, mechanical apheresis using a continuous flow cell separator. For example, lymphocytes and monocytes can be isolated from the buffy coat by any known method, including, but not limited to, separation over Ficoll-Hypaque™ gradient, separation over a Percoll gradient, or elutriation. The concentration of Ficoll-Hypaque™ can be adjusted to obtain the desired population, for example, a population enriched in T cells. Other methods based on affinity are known and can be used. These include, for example, fluorescence-activated cell sorting (FACS), cell adhesion, magnetic bead separation, and the like. Affinity-based methods may utilize antibodies, or portions thereof, which are specific for cell-surface markers and which are

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available from a variety of commercial sources, including, the American Type Culture Collection (Manassas, MD). Affinity-based methods can alternatively utilize ligands or ligand analogs, of cell surface receptors.

The effector cell population can be subjected to one or more separation protocols based on the expression of cell surface markers. For example, the cells can be subjected to positive selection on the basis of expression of one or more cell surface polypeptides, including, but not limited to, "cluster of differentiation" cell surface markers such as CD2, CD3, CD4, CD8, TCR, CD45, CD45RO, CD45RA, CD11b, CD26, CD27, CD28, CD29, CD30, CD31, CD40L; other markers associated with lymphocyte activation, such as the lymphocyte activation gene 3 product (LAG3), signaling lymphocyte activation molecule (SLAM), T1/ST2; chemokine receptors such as CCR3, CCR4, CXCR3, CCR5; homing receptors such as CD62L, CD44, CLA, CD146, a4b7, aEb7; activation markers such as CD25, CD69 and OX40; and lipoglycans presented by CD1. The effector cell population can be subjected to negative selection for depletion of non-T cells and/or particular T cell subsets. Negative selection can be performed on the basis of cell surface expression of a variety of molecules, including, but not limited to, B cell markers such as CD19, and CD20; monocyte marker CD14; the NK cell marker CD56.

An effector cell population can be manipulated by exposure, *in vivo* or *in vitro*, to one or more biological modifiers. Suitable biological modifiers include, but are not limited to, cytokines such as IL-2, IL-4, IL-10, TNF-α, IL-12, IFN-γ; non-specific modifiers such as phytohemagglutinin (PHA), phorbol esters such as phorbol myristate acetate (PMA), concanavalin-A, and ionomycin; antibodies specific for cell surface markers, such as anti-CD2, anti-CD3, anti-IL2 receptor, anti-CD28; chemokines, including, for example, lymphotactin. The biological modifiers can be native factors obtained from natural sources, factors produced by recombinant DNA technology, chemically synthesized polypeptides or other molecules, or any derivative having the functional activity of the native factor. If more than one biological modifier is used, the exposure can be simultaneous or sequential.

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The present invention provides compositions comprising immune effector cells, which may be T cells, enriched in antigen-specific cells, specific for a peptide of the invention. By "enriched" is meant that a cell population is at least about 50-fold, more preferably at least about 500-fold, and even more preferably at least about 5000-fold or more enriched from an original naive cell population. The proportion of the enriched cell population which comprises antigen-specific cells can vary substantially, from less than 10% up to 100% antigen-specific cells. If the cell population comprises at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90%, antigen-specific immune effector cells, specific for a peptide of the invention, then the population is said to be "substantially pure." The percentage which are antigen-specific can readily be determined, for example, by a ^{3}H-thymidine uptake assay in which the effector cell population (for example, a T-cell population) is challenged by an antigen-presenting matrix presenting an antigenic peptide of the invention.

Compositions of the invention

This invention also provides compositions containing any of the above-mentioned peptides, polypeptides, polynucleotides, antigen-presenting matrices, vectors, cells, antibodies and fragments thereof, and an acceptable solid or liquid carrier. When the compositions are used pharmaceutically, they are combined with a "pharmaceutically acceptable carrier" for diagnostic and therapeutic use. These compositions also can be used for the preparation of medicaments for the diagnostic and immunomodulatory methods of the invention.

Methods using the peptides, polynucleotides, antigen-presenting matrices, and host cells of the invention

The present invention provides diagnostic and immunomodulatory methods using peptides, polynucleotides, antigen-presenting matrices, and host cells (including APCs and educated immune effector cells), i.e., immunomodulatory agents, of the invention.

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Diagnostic methods

The present invention provides diagnostic methods using synthetic antigenic peptide epitopes of the invention. The methods can be used to detect the presence of an antigen-specific $CD4^+$ or $CD8^+$ T cell which binds a synthetic antigenic peptide epitope of the invention. Such a T cell is expected to also bind a natural counterpart to the synthetic peptide.

The diagnostic methods of the invention include: (1) assays to predict the efficacy of a synthetic antigenic peptide epitope of the invention; (2) assays to determine the precursor frequency (i.e., the presence and number of) of immune effector cells specific for a synthetic antigenic peptide epitope of the invention and/or its natural counterpart; and (3) assays to determine the efficacy of a synthetic antigenic epitope of the invention once it has been used in an immunomodulatory method of the invention.

Diagnostic methods of the invention are generally carried out under suitable conditions and for a sufficient time to allow specific binding to occur between a synthetic antigenic epitope of the invention and an immune effector molecule, such as a TCR, on the surface of an immune effector cell, such as a CD4+ or CD8+ T cell. "Suitable conditions" and "sufficient time" are generally conditions and times suitable for specific binding. Suitable conditions occur between about 4°C and about 40°C, preferably between about 4°C and about 37°C, in a buffered solution, and within a pH range of between 5 and 9. A variety of buffered solutions are known in the art, can be used in the diagnostic methods of this invention, and include, but are not limited to, phosphate-buffered saline. Sufficient time for binding and response will generally be between about 1 second and about 24 hours after exposure of the sample to the synthetic antigenic peptide epitope of the invention.

In some embodiments, the invention provides diagnostic ssays to predict the efficacy of a synthetic antigenic peptide epitope of the invention. In some of these embodiments, defined T cell epitopes are used to clinically characterize tumors and viral pathogens in order to determine, in advance, the predicted

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efficacy of an *in vivo* vaccine trial. This can be achieved by a simple proliferation assay of a patient's peripheral blood mononuclear cells using defined T cell epitopes as stimulators. Peptides which elicit a response are viable vaccine candidates for that patient.

In other embodiments, assays are provided to determine the precursor frequency (i.e., the presence and number of) of resting (naïve) immune effector cells specific for a synthetic antigenic peptide epitope of the invention and/or its natural counterpart, and which therefore have the potential to become activated. In these embodiments, an antigen-presenting cell bearing on its surface a natural counterpart of a synthetic antigenic peptide epitope of the invention is used to detect the presence of immune effector cells in a biological sample which bind specifically to the natural epitope. A functional assay is used to determine (and quantitate) the antigen-specific immune effector cells. As an illustrative example, PBMCs are isolated from a subject with a tumor. A sample of these PBMCs is cultured together for a suitable time with the tumor cells from the same subject. A second sample of these PBMCs is cultured together for a suitable time with surrogate APCs pulsed with a synthetic antigenic peptide epitope of the invention which is designed to be the synthetic counterpart to a natural epitope expressed on the surface of the tumor. Both tumor cells and surrogate APCs are loaded with ^{51}Cr. By comparing the amount of ^{51}Cr release from the tumor cell and the antigen-pulsed surrogate APC, one can determine the precursor frequency of immune effector cells which are specific for tumor and the precursor frequency of immune effector cells which are specific for the synthetic antigenic peptide epitope. Functional assays include, but are not limited to, immune effector cell proliferation, cytokine production, specific lysis of an APC.

In other embodiments, the efficacy of an immunomodulatory method, including immunomodulatory methods of the invention, in modulating an immune response to a synthetic antigenic epitope of the invention and/or its natural counterpart, can be tested using diagnostic assays of the invention. These diagostic assays are also useful to assess or monitor the efficacy of an immunotherapeutic agent. In some of these embodiments, the method allows

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detection of immune effector cells, which may be activated $CD4^+$ or $CD8^+$ T cells, which have become activated or anergized as a result of exposure to a synthetic antigenic peptide epitope of the invention. A sample containing cells from a subject can be tested for the presence of CD4+ or CD8+ T cells which have become activated or anergized as a result of binding to a given synthetic antigenic peptide epitope of the invention. In some embodiments, the method comprises the steps of: (a) contacting an immobilized antigen-presenting matrix which presents a synthetic antigenic peptide epitope of the invention on its surface bound to a Class I or Class II MHC molecule with a biological sample under suitable conditions and for a time sufficient to allow binding of an immune effector cell which bears on its surface an antigen receptor specific for the peptide, thereby immobilizing the antigen-specific immune effector cell; and (b) contacting the immobilized immune effector cell with a detectably labeled molecule, such as an antibody, which specifically binds the immune effector cell. In other embodiments, the method comprises the steps of (a) contacting an immobilized antigen-presenting matrix which presents a synthetic antigenic peptide epitope of the invention on its surface bound to a Class I or Class II MHC molecule with a biological sample under suitable conditions and for a time sufficient to allow binding of an immune effector cell which bears on its surface an antigen receptor specific for the peptide, thereby immobilizing the antigen-specific immune effector cell; and (b) performing a functional assay on the immobilized immune effector cell. An immobilized antigen-presenting matrix can be a synthetic antigen-presenting matrix immobilized on a solid support including, but not limited to, plates, chips, and beads. Once the immune effector cell is bound to the immobilized synthetic antigenic peptide epitope of the invention, it can be labeled on the basis of characteristic cell surface molecules, including, but not limited to, CD4, CD8, and cell surface markers specific for activated T cells. A variety of cell surface markers specific to populations of immune effector cells are known to those skilled in the art and have been described in numerous publications. see, for example, THE LEUKOCYTE ANTIGEN FACTS BOOK, Barclay et al., eds., 1995, Academic Press. Antibodies to these markers are commercially available from,

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inter alia, Beckman Coulter. The immobilized immune effector cell can also be characterized by presence of mRNA and/or proteins in the cytosol which are characteristic of a given T cell type in a given activated or anergic state. A characteristic mRNA can be detected by any known means, including, but not limited to, a polymerase chain reaction. A detectably labeled antibody to a cell surface marker can be contacted with the immobilized immune effector cell under suitable conditions and for a time sufficient to allow specific binding. If necessary or desired, the labeled cells can be physically removed from unbound label or excess unbound label can be inactivated. The requirements of an antibody specific for a cell surface marker on an immune effector cell are that the antibody bind specifically and that the antibody not interfere with binding between a TCR and the immobilized synthetic antigenic peptide epitope.

Labels which may be employed are known to those skilled in the art and include, but are not limited to, traditional labeling materials such as fluorophores, radioactive isotopes, chromophores, and magnetic particles. Enzyme labels include, but are not limited to, luciferase; a green fluorescent protein (GFP), for example, a GFP from *Aequorea victoria*, or any of a variety of GFP known in the art; α-galactosidase, chloramphenicol acetyl transferase. See, for example, Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987, and periodic updates). Any assay which detects the label, either by directly or indirectly, is suitable for use in the present invention. Assays include colorimetric, fluorimetric, or luminescent assays, radioimmunoassays or other immunological assays.

Immunomodulatory methods

The invention provides methods of modulating an immune response in an individual to a synthetic antigenic peptide epitope of the invention, and thus to the corresponding natural epitope. Immunomodulatory methods of the invention include methods that result in induction or increase, as well as methods that result in suppression or reduction, of an immune response in a subject, and comprise administering to the subject an effective amount of a peptide (or any

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immunomodulatory agent) of the invention in formulations and/or under conditions that result in the desired effect on an immune response (or lack thereof) to the peptide. Immunomodulatory methods of the invention include vaccine methods, adoptive immunotherapy, and methods to induce T cell unresponsiveness, or anergy.

An "immunomodulatory agent" for use in the methods of the invention is a molecule, a macromolecular complex, or a cell that modulates an immune response and encompasses: a synthetic antigenic peptide of the invention alone or in any of a variety of formulations described herein; a polypeptide comprising a synthetic antigenic peptide of the invention; a polynucleotide encoding a peptide or polypeptide of the invention; a synthetic antigenic peptide of the invention bound to a Class I or a Class II MHC molecule on an antigen-presenting matrix, including an APC and a synthetic antigen-presenting matrix (in the presence or absence of co-stimulatory molecule(s)); a synthetic antigenic peptide of the invention covalently or non-covalently complexed to another molecule(s) or macromolecular structure; and an educated, antigen-specific immune effector cell which is specific for a peptide of the invention.

Various methods are known to evaluate T cell activation. CTL activation can be detected by any known method, including but not limited to, tritiated thymidine incorporation (indicative of DNA synthesis), and examination of the population for growth or proliferation, e.g., by identification of colonies. Alternatively, the tetrazolium salt MTT (3-(4,5-dimethyl-thazol-2-yl)-2,5-diphenyl tetrazolium bromide) may be added. Mossman (1983) J. Immunol. Methods **65**:55-63; Niks and Otto (1990) J. Immunol. Methods **130**:140-151. Succinate dehydrogenase, found in mitochondria of viable cells, converts the MTT to formazan blue. Thus, concentrated blue color would indicate metabolically active cells. In yet another embodiment, incorporation of radiolabel, e.g., tritiated thymidine, may be assayed to indicate proliferation of cells. Similarly, protein synthesis may be shown by incorporation of ^{35}S-methionine. In still another embodiment, cytotoxicity and cell killing assays, such as the classical chromium release assay, may be employed to evaluate epitope-specific CTL activation. To

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detect activation of CD4+ T cells, any of a variety of methods can be used, including, but not limited to, measuring cytokine production; and proliferation, for example, by tritiated thymidine incorporation

Release of ^{51}Cr from labeled target cells is a standard assay which can be used to assess the number of peptide-specific CTLs in a biological sample. Tumor cells, or APCs of the invention, are radiolabeled as targets with about 200 μCi of $Na_2\,^{51}CrO_4$ for 60 minutes at 37° C, followed by washing. T cells and target cells (~1 × 10^4/well) are then combined at various effector-to-target ratios in 96-well, U-bottom plates. The plates are centrifuged at 100 × g for 5 minutes to initiate cell contact, and are incubated for 4-16 hours at 37°C with 5% CO_2. Release of ^{51}Cr is determined in the supernatant, and compared with targets incubated in the absence of T cells (negative control) or with 0.1% TRITON™ X-100 (positive control). See, e.g., Mishell and Shiigi, eds. Selected Methods in Cellular Immunology (1980) W.H. Freeman and Co.

The formulation of a peptide of the invention will vary, depending on the desired result. In general, peptides presented on an antigen-presenting matrix by a Class I or Class II MHC molecule, together with the appropriate co-stimulatory molecules, will result in induction of an immune response to the peptide. An anergic (or unresponsive) state may be induced in T lymphocytes by presentation of an antigen by an antigen-presenting matrix (which may be an APC) which contains appropriate MHC molecules on its surface, but which lacks the appropriate co-stimulatory molecules. Any of the various formulations described herein can be used.

Polynucleotides of the invention can be administered in a gene delivery vehicle or by inserting into a host cell which in turn recombinantly transcribes, translates and processed the encoded polypeptide. Isolated host cells containing a polynucleotide of the invention in a pharmaceutically acceptable carrier can be combined with appropriate and effective amount of an adjuvant, cytokine or co-stimulatory molecule for an effective vaccine regimen. In some embodiments, the host cell is an APC, such as a dendritic cell. The host cell can be further modified

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by inserting of a polynucleotide coding for an effective amount of either or both of a cytokine a co-stimulatory molecule.

The methods of this invention can be further modified by co-administering an effective amount of a cytokine or co-stimulatory molecule to the subject.

The agents provided herein as effective for their intended purpose can be administered to subjects having a disease to be treated with an immunomodulatory method of the invention or to individuals susceptible to or at risk of developing such a disease. When the agent is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject. Therapeutic amounts can be empirically determined and will vary with the pathology or condition being treated, the subject being treated and the efficacy and toxicity of the therapy.

The amount of a peptide or immune effector cell of the invention will vary depending, in part, on its intended effect, and is ultimately at the discretion of the medical or veterinary practitioner. The factors to be considered include the condition being treated, the route of adminitstration, and nature of the formulation, the mammal's body weight, surface area, age, and general condition and the particular peptide to be administered. A suitable effective dose of peptides of the invention generally lies in the range of from about 0.0001 μmol/kg to about 1000 μmol/kg bodyweight. The total dose may be given as a single dose or multiple doses, e.g., two to six times per day. For example, for a 75 kg mammal (e.g., a human) the dose range would be about 2.25 μmol/kg/day and a typical dose could be about 100 μmol of peptide. If discrete multiple doses are indicated treatment might typically be 25 μmol of a peptide of the invention given up to 4 times per day. In an alternative administrative regimen, peptides of the invention may be given on alternate days or even once or twice a week. A suitable effective dose of an immune effector cell of the invention generally lies in the range of from about 10^2 to about 10^9 cells per administration. Cells can be administered once, followed by monitoring of the clinical response, such as diminution of disease symptoms or tumor mass. Administration may be repeated on a monthly basis, for

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example, or as appropriate. Those skilled in the art will appreciate that an appropriate administrative regimen would be at the discretion of the physician or veterinary practitioner.

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

The agents and compositions of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

More particularly, an agent of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including nasal, topical (including transdermal, aerosol, buccal and sublingual), parenteral (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease or condition being treated.

Vaccines for cancer treatment and prevention

In one embodiment, immunomodulatory methods of the present invention comprise vaccines for cancer treatment. Cancer cells contain many new antigens potentially recognizable by the immune system. Given the speed with which epitopes can be identified, custom anticancer vaccines can be generated for affected individuals by isolating TILs from patients with solid tumors, determining their MHC restriction, and assaying these CTLs against the appropriate library for reactive epitopes. These vaccines will be both treatments for affected individuals as well as preventive therapy against recurrence (or

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establishment of the disease in patients which present with a familial genetic predisposition to it). Inoculation of individuals who have never had the cancer is expected to be quite successful as preventive therapy, even though a tumor antigen-specific CTL response has not yet been elicited, because in most cases high affinity peptides seem to be immunogenic suggesting that holes in the functional T cell repertoire, if they exist, may be relatively rare. Sette et al. (1994) J. Immunol., **153**:5586-5592. In mice, vaccination with appropriate epitopes not only eliminates established tumors but also protects against tumor re-establishment after inoculation with otherwise lethal doses of tumor cells. Bystryn et al. (1993) *Supra*.

Recent advances in vaccine adjuvants provide effective means of administering peptides so that they impact maximally on the immune system. Del-Giudice (1994) *Experientia* **50**:1061-1066. These peptide vaccines will be of great value in treating metastatic tumors that are generally unresponsive to conventional therapies. Tumors arising from the homozygous deletion of recessive oncogenes are less susceptible to elimination by a humoral (antibody) response and would thus be treated more effectively by eliciting a cellular, CTL response.

Vaccines for diseases caused by pathogenic organisms

Synthetic antigenic peptide epitopes of the present invention are also useful in methods to induce (or increase, or enhance) an immune response to a pathogenic organism. These include pathogenic viruses, bacteria, and protozoans.

Viral infections are ideal candidates for immunotherapy. Immunological responses to viral pathogens are sometimes ineffective as in the case of the lentiviruses such as HIV which causes AIDS. The high rates of spontaneous mutation make these viruses elusive to the immune system. However, a saturating profile of CTL epitopes presented on infected cells will identify shared antigens among different serotypes in essential genes that are largely intolerant to mutation which would allow the design of more effective vaccines.

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Adoptive Immunotherapy Methods

The expanded populations of antigen-specific immune effector cells and APCs of the present invention find use in adoptive immunotherapy regimes and as vaccines.

Adoptive immunotherapy methods involve, in one aspect, administering to a subject a substantially pure population of educated, antigen-specific immune effector cells made by culturing naïve immune effector cells with APCs as described above. In some embodiments, the APCs are dendritic cells.

In one embodiment, the adoptive immunotherapy methods described herein are autologous. In this case, the APCs are made using parental cells isolated from a single subject. The expanded population also employs T cells isolated from that subject. Finally, the expanded population of antigen-specific cells is administered to the same patient.

In a further embodiment, APCs or immune effector cells are administered with an effective amount of a stimulatory cytokine, such as IL-2 or a co-stimulatory molecule.

Methods of inducing T cell anergy

Synthetic antigenic peptide epitopes of the present invention are useful in methods to induce T cell unresponsiveness, or anergy. Disorders which can be treated using these methods include autoimmune disorders, allergies, and allograft rejection.

Autoimmune disorders are diseases in which the body's immune system responds against self tissues. They include most forms of arthritis, ulcerative colitis, and multiple sclerosis. Synthetic antigenic peptide epitopes corresponding to endogenous elements that are recognized as foreign can be used in the development of treatments using gene therapy or other approaches. For example, synthetic CTL epitopes, which can act as "suicide substrates" for CTLs that mediate autoimmunity, can be designed as described above. That is to say, peptides which have a high affinity for the MHC allele but fail to activate the TCR

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could effectively mask the cellular immune response against cells presenting the antigen in question. In support of this approach, it is believed that the long latency period of the HIV virus is due to an antiviral immune response and a mechanism by which the virus finally evades the immune system is by generating epitopes that occupy the MHC molecules but do not stimulate a TCR lytic response, inducing specific T cell anergy. Klenerman et al. (1995) Eur. J. Immunol. 25:1927-1931.

In vitro stimulation of T cells through the complex of T cell-antigen receptor and CD3 alone in the absence of other signals, induces T cell anergy or paralysis. T cell activation as measured by interleukin-2 production and proliferation *in vitro* requires both antigenic and co-stimulatory signals engendered by cell to cell interactions among antigen-specific T cells and antigen presenting cells. Various interactions of these CD2 proteins on the T-cell surface with CD58 (LFA-3) proteins and antigen-presenting cells, those of CD11a/CD18 (LFA-1) proteins with CD54 (ICAM-1) proteins and those of CD5 proteins with CD72 proteins can impart such a co-stimulatory signal *in vitro*. Cytokines derived from antigen-presenting cells (e.g., interleukin-1 and interleukin-6) can also provide co-stimulatory signals that result in T-cell activation *in vitro*. The delivery of both antigenic and co-stimulatory signals leads to stable transcription of the interleukin-2 gene and other pivotal T cell-activation genes. The foregoing co-stimulatory signals depend on protein kinase C and calcium. Potent antigen presenting cells express CD80 (B7 and BB1) and other related surface proteins and many T cells express B7 binding proteins, namely CD28 and CTLA-4 proteins. Binding of CD80 by CD28 and CDLA-4 stimulates a T cell co-stimulatory pathway that is independent of protein kinase C and calcium leading to vigorous T cell proliferation. The stimulation of B cells also depends on the interaction between the specific antigen and the cell-surface immunoglobulin. T cell derived cytokines (e.g., interleukins 1 and 4), physical contact between T cells and B cells through specific pairs of receptors and co-receptors, or both, provide the signal or signals essential for B cell stimulation.

Conventional routes of administration are used. A T-cell stimulating or anergy producing amount (or therapeutically effective amount as described above)

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of an immunotherapeutic antigen-superantigen polymer according to the invention is contacted with the target cells. By "T-cell anergy effective amount" is intended an amount which is effective in producing a statistically significant inhibition of a cellular activity mediated by a TCR. This may be assessed *in vitro* using T-cell activation tests. Typically, T-cell anergy or activation is assayed by tritiated thymidine incorporation in response to specific antigen.

One way in which T cell anergy can be induced is to present to a T cell an antigen-presenting matrix which presents a synthetic antigenic peptide epitope of the invention in an MHC Class I or Class II molecule, but which lack co-stimulatory molecules necessary to activate the T cell. For example, a cell other than a normal antigen presenting cell (APC), which has been transfected with MHC antigen to which a selected T cell clone is restricted, can be used. Resting T cells are provided with an appropriate peptide recognized by the resting T cells in the context of the MHC transfected into a cellular host other than an APC. The MHC is expressed as a result of introduction into a mammalian cell other than an antigen presenting cell of genes constitutively expressing the α and β chains of the MHC class II, or an MHC Class I molecule together with invariant chain. Importantly, these cells do not provide other proteins, either cell surface proteins or secreted proteins, associated with antigen presenting cells, which together with the MHC and peptide result in co-stimulatory signals.

To determine whether anergy has been induced, the T cells to be tested can be cultured together with an antigen presenting matrix which presents a synthetic antigenic peptide epitope of the invention in an MHC Class I or Class II molecule together with co-stimulatory molecules necessary to activate the T cell. The cultures are incubated for about 48 hours, then pulsed with tritiated thymidine and incorporation measured about 18 hours later. The absence of incorporation above control levels, where the T-cells are presented with antigen presenting cells which do not stimulate the T cells, either due to using an MHC to which the T cells are not restricted or using a peptide to which the T cells are not sensitive, is indicative of an absence of activation. One may use other conventional assays to determine the extent of activation, such as assaying for IL-2, -3, or -4, cell surface proteins

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associated with activation, e.g. CD71 or other convenient techniques. Another method is to determine the expression of a protein which is expressed on quiescent T cells, but not on anergic T cells. U.S. Patent No. 5,747,299.

The following examples are intended to illustrate, but not limited to the present invention.

EXAMPLES

EXAMPLE 1

The SPHERE method described above was used to screen from about 5 million peptides (from a library of about 47 million peptides) for epitopes that react with an HLA-A2-restricted $CD8^+$ T cell, TIL1520, which has known specificity for the 209-217 peptide of human gp100. The synthetic 9-mer library was designed with a fixed high-affinity HLA-A2 agretope and a variable TCR epitope repertoire (sequence F-L-X-X-X-X-X-X-V, SEQ ID NO:13 wherein X is any one of 20 amino acids. In an additional embodiment, X is not cysteine). The library de-convolution strategy involved 3 iterations starting with pools of 10,000 peptides for the primary screen.

Several peptide sequences were found which did not correspond to gp100 (209-217). These are shown in Table 1.

TABLE 1.

Amino acid sequences of IGF-II-R synthetic peptides

SEQ ID NO	Amino Acid Sequence
SEQ ID NO:1	F L F S W Y A X V
SEQ ID NO:3	F L F S W F A L V
SEQ ID NO:5	F L Y S W W A I V
SEQ ID NO:7	F L Y S W W W P V
SEQ ID NO:9	F L F L W F F E V
SEQ ID NO:11	Y L F S W Y T S A

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A consensus sequence can be derived from these sequences, the consensus sequence being $FLX_1X_2WX_3X_4X_5V$ (SEQ ID NO:13), wherein X_1 is F or Y or other amino acid, X_2 is S or L or other amino acid, X_3 is an amino acid having an aromatic side chain, X_3 and X_4 are each any amino acid. In an additional embodiment, X is not cysteine.

A BLAST search was performed to identify the natural counterpart of these peptides. It was surprisingly found that some of the peptides had sequence similarity with a mouse IGF-II-R cDNA. BLAST searches showed significant homology to a 9-amino acid stretch of IGF-II-R having the sequence YLFSWYTSA (SEQ ID NO:30), corresponding to amino acids 2268-2276. This 9-amino acid sequence is predicted to be an HLA-A2-restricted peptide with a dissociation half-time of 126.833 minutes. There are several other related sequences within the IGF-II-R protein; however, only the 2268-2276 peptide is predicted to be a good HLA-A2 binder.

MHC binding affinities were calculated using the method described in Parker, K.C. et al. (1992) J. Immunol. **149**(11):3580-3587. The calculated binding affinities are shown in Table 2.

TABLE 2.

Calculated binding affinities of synthetic peptides for HLA-A2.

SEQ ID NO:	Amino Acid Sequence	1/2-time for dissociation (min.)	Fold increase over native sequence
1	F L F S W Y A X V	1775.663	14.00
3	F L F S W F A L V	2249.173	17.73
5	F L Y S W W A I V	713.592	5.63
7	F L Y S W W W P V	13053.519	102.91
9	F L F L W F F E V	13045.201	102.85
11	Y L F S W Y T S A	126.833	1.00

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Since it was known that Hurley R100 T cell population is specific for melanoma antigens, and since the inventors discovered that this cell population also contained cells which are cytotoxic toward IGF-II-R-bearing tumor cells, a search was performed for tumor cells which express both known melanoma antigens and IGF-II-R on their surface. SAGE analysis was performed to determine the relative levels of gp100 and IGF-II-R in these various cell lines. The SAGE method has been described. Velculescu et al. (1995) Science 270:484-487. The results are shown in Table 3.

TABLE 3.

Expression patterns of IGF-IIR and Known Melanoma Antigens Represented in the Legacy Database.

Antigen	A375	Mel 624	Mel 1300	SK-28	SK-131	BA1	NM455	Average Normal
M-6-PR	3	0	0	9	2	0	0	3
GP100	0	466	218	14	0	0	2	350
MART-1	21	18	11	14	0	0	2	40
ESO	12	3	2	0	0	12	5	0
Tyrosinase	0	18	9	5	0	3	0	80
MAGE-2	0	0	2	0	0	0	2	0
MAGE 3,6	3	3	0	21	5	9	7	0
MAGE-8,12	3	0	0	2	0	0	5	0

As a corroborative assay, Northern blot analysis was performed on A375, 624, and 1300 cell lines. The results, shown in Figure 1, demonstrated that A375, unlike 624 and 1300, does not express gp100.

Having established that the cell line A375 expresses IGF-II-R, but not gp100, on its surface, it was determined whether TIL1520 cells are cytotoxic toward A375 cells, a CTL assay was performed, as shown in Figure 2. Target cells (A375) were loaded with ^{51}Cr and irradiated. Effector cells (Hurley R100) were then added at various effector to target cell ratios, and lysis was measured by counting chromium release. As expected, 1300 and 624 cell lines, both of which

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express gp100 on their surfaces, are efficiently lysed. The results indicate that TIL1520 cells comprise a population which specifically lyse A375 cells.

In this manner, peptide sequences that are preferentially recognized by IGF-II-R-specific CTLs were identified and can be used for vaccines having the unique property of being able to reverse peripheral tolerance.

EXAMPLE 2.

Again, the inventor screened 5 million peptides (from a library of about 47 million peptides) for epitopes that react with an HLA-A2-restricted $CD8^+$ T cell with known specificity for the 209-217 peptide of human gp100. The synthetic 9-mer library was designed with a fixed high-affinity HLA-A2 agretope and a variable TCR epitope repertoire (sequence F-L-X-X-X-X-X-X-V (SEQ ID NO:13), wherein X is any one of 20 amino acids. In one embodiment, X is not cysteine. The library de-convolution strategy involved 3 iterations starting with pools of 10,000 peptides for the primary screen. In this manner, peptide sequences that are preferentially recognized by the gp100 209 peptide-specific CTL were identified and can be used for vaccines having the unique property of being able to reverse peripheral tolerance.

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Synthetic peptides corresponding to a gp100 epitope are summarized in Table 4.

TABLE 4

Synthetic gp100 peptide sequences

SEQ ID NO	Amino Acid Sequence
SEQ ID NO:15	F L D Q V A F X V
SEQ ID NO:17	F L D Q V A F V V
SEQ ID NO:19	I T D Q V P F S V
SEQ ID NO:21	F L D Q V P F S V
SEQ ID NO:23	F L D Q R V F F V
SEQ ID NO:25	F L D Q R V F V V
SEQ ID NO:27	F L D Q X X F X V

MHC binding affinities were calculated using the method described in Parker, et al. (1992) J. Immunol. 149(11):3580-3587. The calculated binding affinities are shown in Table 5.

TABLE 5

Calculated binding affinities of peptides for HLA-A2

SEQ ID NO:	Amino Acid Sequence	1/2-time for dissociation (minutes)	Fold increase over native sequence
15	F L D Q V A F X V	742.259	194.82
19	I T D Q V P F S V	3.810	1.00
21	F L D Q V P F S V	742.259	194.82
23	F L D Q R V F F V	9389.576	2464.46
25	F L D Q R V F V V	699.950	183.71

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C57BL/6 mice can be used as an appropriate animal model to assay the use of the above compositions and methods in combination with other known or yet undiscovered anti-tumor therapies. Animals are immunized with the peptides with an intravenous injection of 5 X 10^5 bone marrow-derived dendritic cells (DCs) presenting an antigenic peptides of this invention. DCs not presenting the peptide can serve as a negative control. Two weeks after immunization, the mice are challenged with a subcutaneous injection of 2 X 10^4 tumor cells and tumor growth is monitored over time.

Dendritic cells derived from peripheral blood of a subject such as a human patient are transduced with adenovirus vector containing the polynucleotides of this invention at a multiplicity of infection of 200-500 for use in adoptive immunotherapy. Approximately 24 hours after infection, the transfected dendritic cells (1-5 x10^7 cells) are administered to the patient IV or subcutaneously. The process is repeated 3-4 weeks later with up to 5 administrations of dendritic cells. Since it is possible to freeze dendritic cells and administer thawed cells, the subject does not have to be leukapheresed each time.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

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CLAIMS

What is claimed is:

1. An isolated polynucleotide encoding a polypeptide comprising the sequence FLFSWYAXV (SEQ ID NO:1), wherein X is any amino acid.

2. The isolated polynucleotide of claim 1, wherein X is any amino acid, excluding cysteine.

3. An isolated polynucleotide encoding a polypeptide comprising the sequence FLFSWFALV (SEQ ID NO:3).

4. An isolated polynucleotide encoding a polypeptide comprising the sequence FLYSWWAIV (SEQ ID NO:5).

5. An isolated polynucleotide encoding a polypeptide comprising the sequence FLYSWWWPV (SEQ ID NO:7).

6. An isolated polynucleotide encoding a polypeptide comprising the sequence FLFLWFFEV (SEQ ID NO:9).

7. An isolated polynucleotide encoding a polypeptide comprising the sequence FLXXWXXXV (SEQ ID NO:13), where X is any amino acid.

8. The isolated polynucleotide of claim 7, wherein X is any amino acid excluding cysteine.

9. A polynucleotide encoding a polypeptide comprising the sequence FLDQVAFXV (SEQ ID NO:15), wherein X is any amino acid.

10. The isolated polynucleotide of claim 9, wherein X is any amino acid excluding cysteine.

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11. A polynucleotide encoding a polypeptide comprising the sequence FLDQVAFVV (Seq. ID No. 17).

12. A polynucleotide encoding a polypeptide comprising the sequence FLFSWYAXP (SEQ ID NO:19) wherein X is any amino acid.

13. The isolated polynucleotide of claim 12, wherein X is any amino acid excluding cysteine.

14. A polynucleotide encoding a polypeptide comprising the sequence FLDQVPFSV (SEQ ID NO:21).

15. A polynucleotide encoding a polypeptide comprising the sequence FLDQRVFFV (SEQ ID NO:23).

16. A polynucleotide encoding a polypeptide comprising the sequence FLDQRVFVV (SEQ ID NO:25).

17. A polynucleotide encoding a polypeptide comprising the sequence FLDQXXFXV (SEQ ID NO:27), wherein X is any amino acid.

18. The isolated polynucleotide of claim 17, wherein X is any amino acid, excluding cysteine.

19. The complement of a polynucleotide of any of claims 1 to 18.

20. A gene delivery vehicle comprising a polynucleotide of any one of claims 19.

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21. An isolated host cell comprising a polynucleotide of any one of claims 1-18.

22. An isolated host cell comprising a polynucleotide of claim 19.

23. A composition comprising the polynucleotide of any of claims 1-18.

24. A composition comprising a polynucleotide of claim 19.

25. The polypeptide encoded by the polynucleotide of any of claims 1-18.

26. The host cell of claim 22, wherein the cell is an antigen presenting cell (APC) and the peptide is presented by a Class I MHC molecule on the surface of the cell.

27. The host cell of claim 26, wherein the antigen presenting cell (APC) is a dendritic cell.

28. A peptide encoded by a polynucleotide of any one of claims 1-8.

29. A non-naturally occurring peptide synthesized by the host cell of claim 21.

30. A method of producing a population of educated, antigen-specific immune effector cells, comprising culturing naïve immune effector cells with an antigen-presenting matrix which presents the peptide produced by a polynucleotide of any one of claims 1-18, on its surface bound to a Class I MHC molecule under conditions and for a suitable time to allow activation of an immune effector cell which bears on its surface an antigen receptor specific for the peptide.

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31. A population of educated, antigen-specific immune effector cells produced by culturing naïve immune effector cells with antigen presenting cells (APCs) wherein the peptide is encoded by the polynucleotide of any of claims 1-18, is presented by a Class I MHC molecule on the surface of the APCs.

32. A method of inducing an immune response in an individual, comprising administering to the individual an effective amount of a polynucleotide of any of claims 1-18, under conditions that induce an immune response to the polypeptide.

33. A method of inducing an immune response in an individual, comprising administering to the individual an effective amount of the peptide encoded by the polynucleotide of any one of claims 1 to 18, under conditions that induce an immune response to a polypeptide encoded by the polynucleotide.

34. A method of inducing an immune response to a tumor antigen in an individual, comprising administering to the individual an effective amount of the cell of claim 26 under conditions that induce an immune response to the antigen.

35. A method of adoptive immunotherapy, comprising administering to an individual an effective amount of a population of educated, antigen-specific immune effector cells of claim 31.

36. A method of detecting an antigen-specific immune effector cell comprising the steps of:

(a) contacting an immobilized antigen-presenting matrix which presents a peptide encoded by a polynucleotide of any of claims 1-18 on its surface bound to a Class I MHC molecule with a biological sample under conditions and for a suitable time to allow binding of an immune effector cell which bears on its

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surface an antigen receptor specific for the peptide, thereby immobilizing the antigen-specific immune effector cell; and

(b) contacting the immobilized immune effector cell with a detectably labeled molecule which specifically binds the immune effector cell

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37. An array of probes comprising a polynucleotide of any one of claims 1 to 18 attached to a chip.

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FIGURE 1

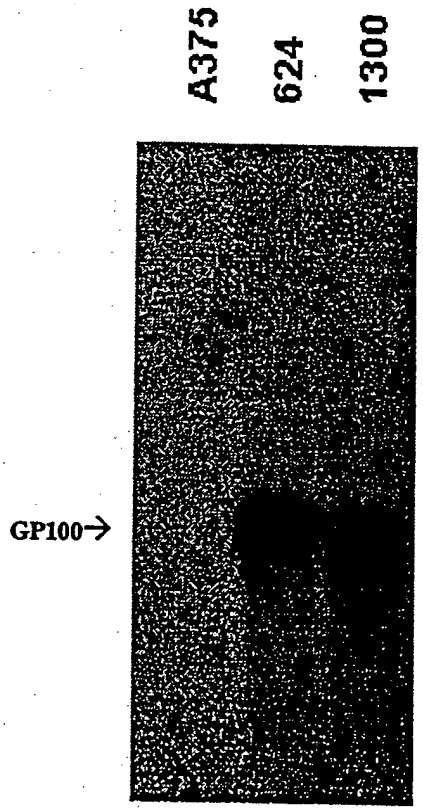
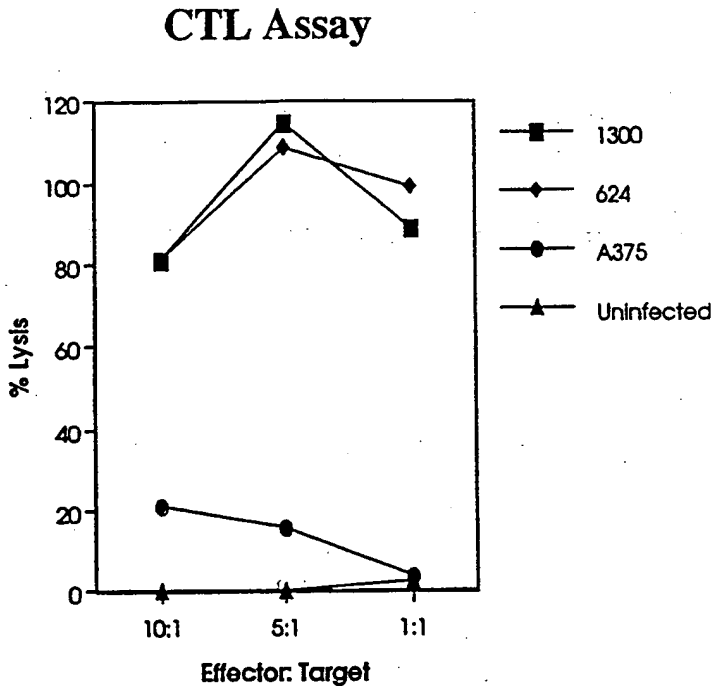


FIGURE 2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SEQUENCE LISTING

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Nicolette, Charles A.

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BNSDOCID: <WO___0020457A1_I_>

INTE...ATIONAL SEARCH REPORT

Int... ional Application No
PCT/US 99/23167

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/705 C12N15/12 G01N33/50 A61K39/00 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 702 082 A (HAGIWARA, Y.) 20 March 1996 (1996-03-20) * claim 1, item (3); claim 6, item (3); pages 1-2 *	7,25,28, 32-34
X	COLONNA, M. & SAMARIDIS, J.: "Cloning of immunoglobulin-superfamily members..." SCIENCE, vol. 268, 1995, pages 405--408, XP002067321 * fig. 2; abstract *	7,25,28, 32-34
X	COCKLE, S.M. ET AL.: "Thyrotrophin-releasing hormone-related polypeptides ..." J. ENDOCRINOL., vol. 120, 1989, pages 31-36, XP000872489 * page 32, first paragraph *	7,25,28

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 2000	03.03.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hermann, R

1

Form PCT/ISA/210 (second sheet) (July 1992)

BNSDOCID: <WO___0020457A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/23167

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 29,31,35
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BNSDOCID: <WO___0020457A1_I_>

INTE..ATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 99/23167

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 702082 A	20-03-1996	JP 7101999 A	18-04-1995
		US 5589573 A	31-12-1996

Form PCT/ISA/210 (patent family annex) (July 1992)

BNSDOCID: <WO___0020457A1_I_>

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 29,31,35

The peptides of claim 28 and the cells of claim 31 are not characterised by unequivocal technical features:
A peptide is not characterised by a reference to its origin, and a population of cells cell is not sufficiently characterised by a reference to its production. Kown compounds or cells are not rendered novel by a new origin, or a new method for obtaining them.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.